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Kongeriget Danmark

Patent application No.: 0506/98

Date of filing: 08 Apr 1998

Applicant: Novo Nordisk A/S, Novo Allé, Bagsværd, DK

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TAASTRUP 17 Feb 1999

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TITLE:

An enzymatic oil-degumming process

FIELD OF INVENTION

The present invention relates to an improved process for enzymatic reducing the content of phosphorus containing components in an edible oil.

BACKGROUND OF THE INVENTION

Oils obtained from the usual oil and fat production processes by compressing oil-bearing materials or by extracting oil from the materials and removing the extraction solvent contain impurities such as polar lipids mainly composed of phospholipids, as well as fatty acids, pigments, odor components and the like. Thus it is necessary to remove these impurities by a refining process. Such a process may require a degumming step.

In the art it is known to use phospholipase for enzymatic degumming of an edible oil (US 5,264,367; JP-A-2153997; and EP 622446), to reduce the phosphorus content of said water degummed edible oil.

However those references do not specifically suggest to use low amount of water in the enzymatic degumming process.

In contrary EP 622446 suggest to use high amount of water in the enzymatic degumming process. See page 3, line 33-44 and claim 4 in said document, which suggest to use more than 30 percent of water by weight of the oil in said process.

SUMMARY OF THE INVENTION

The problem, to be solved, by the present invention is to provide a simplified and economically cheaper process for enzymatic degumming of edible oils.

The solution is to perform said process using low amounts of water.

Accordingly, the present invention relates to a process for reducing the content of phosphorus containing components in an edible oil, having from 50 to 10.000 part per million (ppm) of phosphorous content, which method comprises contacting said oil at a pH from 1.5 to 8 with an aqueous solution of a

phospholipase A1 (PLA1), phospholipase A2 (PLA2), or phospholipase B (PLB) which is emulsified in the oil until the phosphorous content of the oil is reduced to less than 12 ppm, and then separating the aqueous phase from the treated oil, and wherein said process is characterized by that said emulsified condition is formed using from 0.01 to 1.5 percent of water by weight of the oil, preferably from 0.01 to 1.0 percent of water by weight of the oil, and most preferably from 0.01 to 0.5 percent of water by weight of the oil.

An advantage of the process described herein is that costs for water and waste water treatment may be reduced. Furthermore, oil recovery yields may be increased because less amount of oil will be wasted to the aqueous phase.

Further, an advantage of the process described herein may be that an oil-mill using this process may skip sludge recycling of the polluted water used in the process.

The in the art known enzymatic degumming processes give rise to a high amount of polluted water, which is expensive to clean up. This is of course an economically burden.

Further oil-mills traditionally have been forced to implement recycling of the water processes in order to save cost in said purifying of the polluted water.

Said recycling step may be saved by the low amount of water used in the process described herein.

In enzymatic degumming carried out according to the art (e.g. US 5,264,367) a heat treatment to e.g. 65-75 °C of the water in oil emulsion is usually carried out in order to facilitate separation of the oil and aqueous phases by e.g. centrifugation. When using the thermostable phospholipsae Lecitase™ (Novo Nordisk A/S, Denmark) in the oil degumming process, the aqueous phase containing the enzyme can advantageously be reused several times (with or without addition of fresh enzyme solution).

However, for the oil mill it may be advantageous if the recycling of the aqueous phase could be totally omitted. This would in the normal case mean that overall water consumption would be increased with increased costs. If only a low amount of water is used in the enzymatic degumming process, recycling of

the sometimes problematic sludge phase could be omitted.

Embodiment(s) of the present invention is described below, by way of example(s) only.

DETAILED DESCRIPTION OF THE INVENTION

Edible oils:

In principle any edible oil may be degummed according to a process of the invention. Example of oils are crude oils and water degummed oils.

A crude oil (also called a non-degummed oil) may be a pressed or extracted oil or a mixture thereof from e.g. rapeseed, soybean, or sunflower. The phosphatide content in a crude oil may vary from 0.5-3% w/w corresponding to a phosphorus content in the range of 200-10.000 ppm, more preferably in the range of 250-1200 ppm. Apart from the phosphatides the crude oil also contains small concentrations of carbohydrates, sugar compounds and metal/phosphatide acid complexes of Ca, Mg and Fe.

Preferably, said edible oil is an oil from which mucilage has previously been removed and which has a phosphorus content from 50 to 250 ppm.

Such an oil is generally obtained by a water-degumming process and termed "a water-degummed oil".

A water-degummed oil is typically obtained by mixing 1-3% w/w of hot water with warm (60-90°C) crude oil. Usual treatment periods are 30-60 minutes. The water-degumming step removes the phosphatides and mucilaginous gums which become insoluble in the oil when hydrated. The hydrated phosphatides and gums can be separated from the oil by settling, filtering or centrifuging - centrifuging being the more prevalent practice.

Alternatively, the process here termed "water-degumming" may be called "wet refining to remove mucilage" (see US 5,264,367).

Further, an edible is preferably an vegetable oil.

A Phospholipase used in the process:

Preferably, a phospholipase used in the process of the invention is a phospholipase obtained from a microorganism,

preferably a filamentous fungus, a yeast, or a bacterium.

For the purpose of the present invention the term "obtained from", as used herein in connection with a specific microbial source, means that the enzyme and consequently the DNA sequence encoding said enzyme is produced by the specific source.

The enzyme is then obtained from said specific source by standard known methods enabling the skilled person to obtain a sample comprising the enzyme and capable of being used in a process of the invention. Said standard methods may be direct purification from said specific source or cloning of a DNA sequence encoding the enzyme followed by recombinant expression either in the same source (homologous recombinant expression) or in a different source (heterologous recombinant expression).

More preferably, a phospholipase used in a process of the invention is obtained from a filamentous fungal species within the genus *Fusarium*, such as a strain of *F. culmorum*, *F. heterosporum*, *F. solani*, or in particular a strain of *F. oxysporum*; or

a filamentous fungal species within the genus *Aspergillus*, such as a strain of *Aspergillus awamori*, *Aspergillus foetidus*, *Aspergillus japonicus*, *Aspergillus niger* or in particular *Aspergillus oryzae*.

Examples of suitable *Fusarium* phospholipases are disclosed in

i) Tsung-Che et al. (Phytopathological notes 58:1437-38 (1968)) (a phospholipase from *Fusarium solani)*; and

ii) EP Patent Application No. 97610056.0 disclosing a suitable *F. culmorum PL* (see example 18 in said doc.) and a suitable *F. oxysporum PL* (see example 1-17).

Suitable *Aspergillus* phospholipases are diclosed in

i) EP 575133 disclosing numerous different Aspergillus PL`s (see claim 14) and in particular a PL from A. oryzae(Claim 17 or 18) and a PL frcm A. niger (claim 19); and

ii) DE 19527274 A1 dicloses a suitable Aspergillus preparation (see examples).

Further the commercial available phospholipase preparation

Degomma VOD (Roehm, Germany), which is believed to comprise an Aspergillus phospholipase is suitable to be used in a process of the invention.

Further, it is preferred that a phospholipase used in a process of the invention exhibits certain properties.

Accordingly, embodiment of the invention relates to

i) a process according to the invention, wherein the phospholipase is a phospholipase which is substantively independent of Ca^{2+} concentration measured as,

relative phospholipase activity at 5 mM EDTA and 5mM Ca^{2+} in a phospholipase activity assay measuring release of free fatty acids from lecithin in a buffer comprising 2% lecithin, 2% Triton X-100, 20 mM citrate, pH 5; incubated for 10 min. at 37°C followed by stop of reaction at 95°C for 5 min.;
wherein the ratio of relative phospholipase activity at 5mM EDTA/5 mM Ca^{2+} is greater than 0.25, more preferably greater than 0.5; and/or

ii) a process according to the invention, wherein the phospholipase is a phospholipase which has a phospholipase activity which is capable of releasing at least 7 µmol of free fatty acid/min./mg enzyme; more preferably at least 15 µmol of free fatty acid/min./mg enzyme; measured as,

phospholipase activity is measured in an assay measuring release of free fatty acids from lecithin in a buffer comprising 2% lecithin, 2% Triton X-100, 20 mM citrate, pH 5; incubated for 10 min. at 37°C followed by stop of reaction at 95°C for 5 min..

Detailed description of above mentioned assays are disclosed in a working example herein (*vide infra*). For even further details reference is made to EP Patent Application No. 97610056.0 (see example 9 in said document).

Further it has been demonstrated that a phospholipase special suited for enzymatic oil degumming in general and in particular for the improved process described herein is characterized by having a certain primary amino acid sequence.

Accordingly, in an even further embodiment the invention relates to a process according to the invention, wherein the

phospholipase is a phospholipase having an polypeptide sequence selected from the group comprising of:

(a) polypeptide having an amino acid sequence as shown in positions 31-346 of SEQ ID NO 1;

(b) a polypeptide having an amino acid sequence as shown in position 31-303 of SEQ ID NO 1;

(c) a polypeptide which is at least 70 % homologous with said polypeptide defined in (a), or (b); and

a fragment of (a), (b) or (c).

For a detailed description of cloning and purification of a phospholipase having the above mentioned polypeptide sequence reference is made to EP Patent Application No. 97610056.0.

In this document it can further be seen that a phospholipase obtained from *F. oxysporum* and having the polypeptide sequence shown in (b) above exhibits both of the above mentioned functional characteristic. Accordingly, this phospholipase is the most preferred phospholipase to be used in a process of the invention. A working example herein demonstrates the use of this phospholipase (vide infra).

Finally an example of a suitable non-microbial phospholipase is the commercial available PL (Lecitase™, Novo Nordisk A/S, Denmark) obtained from porcine pancreas.

<u>Standard process parameters of the process of the invention:</u>

Besides the specific use of low amount of water in the process of the invention, any of the other process parameters may be done according to the art. See Background section above for references to the art known processes.

The enzymatic treatment is conducted by dispersing an aqueous solution of the phospholipase, preferably as droplets with an average diameter below 10 µ(micro)m.

According to the process of the invention the amount of water is from 0.01 to 1.5% by weight in relation to the oil.

An emulsifier may optionally be added. Mechanical agitation may be applied to maintain the emulsion.

The enzymatic treatment can be conducted at any pH in the range 1.5-8, preferably from pH 3-6. The pH may be adjusted by adding citric acid, a citrate buffer, NaOH or HCl.

A suitable temperature is generally 30-75°C (particularly 40-60°C). The reaction time will typically be 0.5-12 hours (e.g. 2-6 hours), and a suitable enzyme dosage will usually be 100-5000 IU per liter of oil, particularly 200-2000 IU/l.

The enzymatic treatment may be conducted batchwise, e.g. in a tank with stirring, or it may be continuous, e.g. a series of stirred tank reactors.

The enzymatic treatment is followed by separation of an aqueous phase and an oil phase. This separation may be performed by conventional means, e.g. centrifugation. The process of the invention can reduce this value to below 12 ppm, more preferably below 10, and even more preferably below 5 ppm.

MATERIALS AND METHODS

EXAMPLES

EXAMPLE 1

General description of assay for enzymatic degumming of edible oil

Equipment for carrying out enzymatic degumming

The equipment consists of a 1 l jacketed steel reactor fitted with a steel lid, a propeller (about 600 rpm), baffles, a temperature sensor, an inlet tube at the top, a reflux condenser (about 4°C) at the top, and an outlet tube at the bottom. The reactor jacket is connected to a thermostat bath. The outlet tube is connected via silicone tubing to a Silverson in-line mixer head equipped with a "square hole high shear screen", driven by a Silverson L4RT high shear lab mixer (about 8500 rpm, flow ca. 1.1 l/minute). The mixer head is fitted with a cooling coil (5-10 °C) and an outlet tube, which is connected to the inlet tube of the reactor via silicone tubing. A temperature sensor is inserted in the silicone tubing just after the mixer head. The only connection from the reactor/mixer head system to the atmosphere is through the reflux condenser.

General procedure for carrying out enzymatic degumming

All cooling and thermostat equipment is turned on. Then 0.6 l (ca. 560 g) of oil is loaded in the reactor, which is kept at about the temperature needed for the specific experiment. The lab mixer is turned on, whereby the oil starts to circulate from the reactor to the mixer head and back to the reactor. The system is allowed to equilibrate for about 10 minutes, during which period the temperature is fine tuned. The pre-treatment period starts with addition of 0.6 g (2.86 mmol) citric acid monohydrate in the appropriate amount of water or the appropriate amount of a mixture of citric acid and trisodium citrate (see Tables 1 and 7 below; [citric acid] in water/oil emulsion = 4.6 mM), which sets t = 0. At t = 30 minutes the appropriate amount of 4 M NaOH solution is added (see Tables 1 and 7).

Table 1. Water content in Experiments A-D; wdg rape seed oil.

Experiment	Water content	Water in 560 g oil	Water added at t=0	Water in NaOH solution	Water in enzyme solution	Total water
A	[illegible]	0.56 g	27 g	1.1 g	1.0 g	29.7 g
B	[illegible]	0.56 g	5.0 g	0.7 g	1.0 g	7.3 g
C	[illegible]	0.56 g	0.05 g*	0 g	1.0 g	1.6 g
D	[illegible]	0.56 g	0.07 g**	0 g	1.0 g	1.6 g

* Water contribution from o.6 g citric acid monohydrate.

** Water contribution from mixt. of 0.5 g citric acid monohydrate and 0.14 g trisodium citrate dihydrate.

At t = 35 minutes samples are drawn for P-analysis and pH determination. Just after this the required amount of enzyme solution is added (end of pre-treatment period). Samples for P-analysis and pH determination are drawn at t = 1, 2, 3.5, 5, 6 hours, and then the reaction is stopped.

The reactor/mixer system is emptied and rinsed with 2×500

ml 10% Deconex/DI water solution followed by minimum 3×500 ml of DI water. Table 2 is a presentation of the various additions and samplings during the reaction.

Table 2. Schedule for enzymatic degumming

Time	Addition of	Sampling	
		P-analysis	pH determination
		X	
0	Citric acid		
5 min.			X
30 min.		X	X
30 + δ min.	NaOH		
35 min.		X	X
35 + δ min.	Enzyme		
1 hour		X	X
2 hours		X	X
3.5 hours		X	X
5 hours		X	X
6 hours		X	X

Phosphorus analysis:

Sampling for P-analysis:

Take 10 ml of water in oil emulsion in a glass centrifuge tube. Heat the emulsion in a boiling water bath for 30 minutes. Centrifuge at 5000 rpm for 10 minutes. Transfer about 8 ml of upper (oil) phase to a 12 ml polystyrene tube and leave it (to settle) for 12-24 hours. After settling draw about 1-2 g from the upper clear phase for P-analysis.

P-analysis was carried out according to procedure 2.421 in "Standard Methods for the Analysis of Oils, Fats, and Derivatives, 7th ed. (1987)":

Weigh 100 mg of MgO (leicht, Merck #5862) in a porcelain dish and heat with a gas burner. Add 1-2 g of oil and ignite

with a gas burner to give a black, hard mass. Heat in a Vecstar furnace at 850°C for 2 hours to give white ashes. Dissolve the ashes in 5 ml of 6 M HNO_3 and add 20 ml of reagent mix. Leave for 20 minutes. Measure absorbance at 460 nm (use a blank (5 ml HNO_3 + 20 ml reagent mix) for zero adjustment). Calculate by using calibration curve.

pH determination

Take 2 ml of water in oil emulsion and mix with 2 ml of MilliQ water. After phase separation, pipette off top oil layer. Measure pH in aqueous phase with pH electrode Orion. Measurements are transformed to "real" pH values by the formula

$$pH_{real} = pH_{measured} - 0.38.$$

A calibration curve was obtained by dissolving 0.6 g of citric acid monohydrate in 27 g of DI water; pH of this solution was measured by pH electrode Orion (pH_{real}). 100 µl were mixed with 2 ml MilliQ water, and pH of this solution was measured by pH electrode Orion ($pH_{measured}$). pH of the citric acid solution was changed gradually by adding NaOH solution, and for each adjustment dilution and pH measurements were carried out as described above.)

EXAMPLE 2

Degumming of water-degummed rape seed oil (I)

Experiments were carried out according to the "General procedure for carrying out enzymatic degumming" as described in example 1 above.

Oil:

Water-degummed rape seed oil from Århus Oliefabrik (AOM), Denmark. Batches C00730/B01700 and C00730/B01702, P-content 231-236 ppm. Water content ≤ 0.1 % w/w.

Enzyme:

PL from *Fusarium oxysporum* having the amino acid sequence shown in SEQ NO 1.

Batch F-9702027, estimated conc. 0.75 mg/ml.
The enzyme was recombinantly expressed and purified as described in EP Patent application number 97610056.0.

Experiment A (water content 5.3 %)

0.6 l (560 g) of wdg rape seed oil is loaded in the equipment and heated to 40°C. At t = 0 min. a solution of 0.6 g of citric acid monohydrate in 27 g of water was added. At t = 30 min. 1.07 ml (4.3 mmoles) of 4 M NaOH solution were added, which yield a pH of about 5. At t = 35 min., 1 ml (0.75 mg) of a purified solution of phospholipase from *F. oxysporum* is added. The measured phosphorus content in the oil phase after centrifugation as well as the pH values in the aqueous phase is shown in Table 3.

Table 3. Results from degumming of wdg rape seed oil with phospholipase from *F. oxysporum*, water content 5.3 %.

Time (hours)	Phosphorus content in oil phase	pH
0	243	
0.50	215	4.7
0.58	216	5.5
1.0	66	4.9
2.0	10	4.9
3.5	8	5.4
5.0	9	5.0

Experiment B (water content 1.3 %)

As in Experiment A above except that at t = 0 min. 0.6 g of citric acid monohydrate in 5.0 g of water was added, and at t = 30 min. 0.71 ml (2.86 mmoles) of 4 M NaOH solution were added which yield a pH of about 5. The measured phosphorus content in the oil phase after centrifugation as well as the pH values in the aqueous phase is shown in Table 4.

Table 4. Results from degumming of wdg rape seed oil with phospholipase from *F. oxysporum*, water content 1.3 %.

Time (hours)	Phosphorus content in oil phase	pH
0	237	
0.50	213	4.7
0.58	197	5.7
1.0	78	4.9
2.0	9	4.9
3.5	10	5.0
5.0	12	5.1
6.0	10	5.0

Experiment C (water content 0.3 %)

As in Experiment A above except that at t = 0 min. 0.6 g of citric acid monohydrate powder was added, and at t = 30 min. no NaOH solution was added, which yield a pH of about 5. The measured phosphorus content in the oil phase after centrifugation as well as the pH values in the aqueous phase is shown in Table 5.

Table 5. Results from degumming of wdg rape seed oil with phospholipase from *F. oxysporum*, water content 0.3 %.

Time (hours)	Phosphorus content in oil phase	pH
0	246	4.9
0.50	234	5.1
0.58		
1.0	101	4.8

2.0	18	5.2
3.5	11	5.2

Experiment D (water content 0.3 %)

As in Experiment C above except that at t = 0 min. a mixture of 0.5 g of citric acid monohydrate and 0.14 g trisodium citrate dihydrate powder was added, which yield a pH of about 5. The measured phosphorus content in the oil phase after centrifugation as well as the pH values in the aqueous phase is shown in Table 6.

Table 6. Results from degumming of wdg rape seed oil with phospholipase from *F. oxysporum*, water content 0.3 %.

Time (hours)	Phosphorus content in oil phase	pH
0	243	
0.50	244	5.5
0.58		
1.0	101	5.1
2.0	8	4.9

EXAMPLE 3

Degumming of crude (mixture of pressed and extracted) rape seed oil (II)

Experiments were carried out according to the "General procedure for carrying out enzymatic degumming" as described in example 1 above.

Oil:

Crude rape seed oil from MILO Olomouk, Czech rep. Batch C00745/B02042, P-content 263 ppm. Water content 0.17 % w/w.

Table 7. Water content in Experiments E and F; crude rape seed oil.

Experiment	Water content	Water in 560 g oil	Water added at t=0	Water in NaOH solution	Water in enzyme solution	Total water
E	5.4 %	0.95 g	27 g	1.1 g	1.0 g	30.1 g
F	1.4 %	0.95 g	5.0 g	0.7 g	1.0 g	7.7 g

Experiment E (water content 5.4 %)

0.6 l (560 g) of crude rape seed oil is loaded in the equipment and heated to 40°C. At t = 0 min. a solution of 0.6 g of citric acid monohydrate in 27 g of water was added. At t = 30 min. 1.07 ml (4.3 mmoles) of 4 M NaOH solution were added, which yield a pH of about 5. At t = 35 min., 1 ml (0.75 mg) of a purified solution of phospholipase from *F. oxysporum* is added. The measured phosphorus content in the oil phase after centrifugation as well as the pH values in the aqueous phase is shown in Table 8.

Table 8. Results from degumming of crude rape seed oil with phospholipase from *F. oxysporum*, water content 5.4 %.

Time (hours)	Phosphorus content in oil phase	pH
0	222	
0.50	165	
0.58	136	4.8
1.0	38	5.1
2.0	10	5.0
3.5	11	5.0
5.0	11	5.0

6.0	10	5.3

Experiment F (water content 1.4 %)

As in Experiment E above except that at t = 0 min. 0.6 g of citric acid monohydrate in 5.0 g of water was added, and at t = 30 min. 0.71 ml (2.86 mmoles) of 4 M NaOH solution were added which yield a pH of about 5. The measured phosphorus content in the oil phase after centrifugation as well as the pH values in the aqueous phase is shown in Table 9.

Table 9. Results from degumming of crude rape seed oil with phospholipase from *F. oxysporum*, water content 1.4 %.

Time (hours)	Phosphorus content in oil phase	pH
0	223	
0.50	119	
0.58	92	5.1
1.0	31	5.1
2.0	12	5.0
3.5	11	5.1
5.0	9	4.8
6.0	8	4.3

EXAMPLE 4

Assays used for characterization of a phospholipase suitable to be used in an oil degumming process of the invention.

Phospholipase activity assays:

Phospholipase activity (PHLU) was measured as the release of free fatty acids from lecithin. 50 µl 4% L-alpha-phosphatidylcholine (plant lecithin from Avanti, USA), 4% Triton X-100, 5 mM $CaCl_2$ in 50 mM HEPES, pH 7 was added, 50 µl enzyme solution diluted to an appropriate concentration in 50 mM HEPES, pH 7. The samples were incubated for 10 min at 30°C and the

reaction stopped at 95°C for 5 min prior to centrifugation (5 min at 7000 rpm). Free fatty acids were determined using the NEFA C kit from Wako Chemicals GmbH; 25 µl reaction mixture was added to 250 µl reagent A and incubated for 10 min at 37°C. Then 500 µl Reagent B was added and the sample was incubated again, 10 min at 37°C. The absorption at 550 nm was measured using an HP 8452A diode array spectrophotometer. Samples were run at least in duplicates. Substrate and enzyme blinds (preheated enzyme samples (10 min at 95°C) + substrate) were included. Oleic acid was used as a fatty acid standard. 1 PHLU equals the amount of enzyme capable of releasing 1 µmol of free fatty acid/min under these conditions.

Alternatively, the assay was run at 37°C in 20 mM citrate buffer, pH 5 (Ca^{2+}-dependence) or 20 mM Britton-Robinson buffer (pH-profile/temperature-profile/stability).

Phospholipase A1 activity (PLA1) was measured using 1-(S-decanoyl)-2-decanoyl-1-thio-sn-glycero-3-phosphocholine (D3761 Molecular Probes) as a substrate. 190 µl substrate (100 µl D3761 (2 mg/ml in ethanol) + 50 µl 1 % Triton X-100 + 1.85 ml 50 mM HEPES, 0.3 mM DTNB, 2 mM $CaCl_2$, pH 7) in a 200 µl cuvette were added to 10 µl enzyme, and the absorption at 410 nm was measured as a function of time on the HP 8452A diode array spectrophotometer at room temperature. Activity was calculated as the slope of the curve in the linear range. PLA1 equals the amount of enzyme capable of releasing 1 µmol of free fatty acid (thiol)/min at these conditions.

Phospholipase A2 activity (PLA2) was measured at 40°C using 1-hexadecanoyl-2-(1-pyrenedecanoyl)-sn-glycero-3-phosphocholine (H361 Molecular Probes). 2 ml substrate (50 µl 1% Triton X-100 + 25 µl 0.1% H361 in methanol + 10 ml 50mM HEPES, pH 7) in a 2 ml cuvette with stirring was added to 10 µl enzyme, and the pyrene fluorescence emission was measured at 376 nm (excitation at 340 nm) as a function of time (1 sec. intervals) using the Perkin Elmer LS50 apparatus. In the Triton X-100/phospholipid micelles the concentration of phospholipid was adjusted to have excimer formation (emits at 480 nm). Upon cleavage the fatty acid in the 2-position containing the pyrene group is released into the aqueous phase resulting in an increase in the monomer emission. PLA2 was taken as the slope of the curve in the linear range at

equal conditions.

SEQUENCE LISTING

SEQ ID No. 1 shows the amino acid sequence of a phospholipase suitable to be used in an oil-degumming process of the invention.

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 346 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

```
Met Leu Leu Leu Pro Leu Leu Ser Ala Ile Thr Leu Ala Val Ala Ser
 1               5                  10                  15

Pro Val Ala Leu Asp Asp Tyr Val Asn Ser Leu Glu Glu Arg Ala Val
            20                  25                  30

Gly Val Thr Thr Thr Asp Phe Ser Asn Phe Lys Phe Tyr Ile Gln His
        35                  40                  45

Gly Ala Ala Ala Tyr Cys Asn Ser Glu Ala Ala Ala Gly Ser Lys Ile
    50                  55                  60

Thr Cys Ser Asn Asn Gly Cys Pro Thr Val Gln Gly Asn Gly Ala Thr
65                  70                  75                  80

Ile Val Thr Ser Phe Val Gly Ser Lys Thr Gly Ile Gly Gly Tyr Val
                85                  90                  95

Ala Thr Asp Ser Ala Arg Lys Glu Ile Val Val Ser Phe Arg Gly Ser
            100                 105                 110

Ile Asn Ile Arg Asn Trp Leu Thr Asn Leu Asp Phe Gly Gln Glu Asp
        115                 120                 125

Cys Ser Leu Val Ser Gly Cys Gly Val His Ser Gly Phe Gln Arg Ala
    130                 135                 140
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Trp Asn Glu Ile Ser Ser Gln Ala Thr Ala Ala Val Ala Ser Ala Arg
145                 150                 155                 160

Lys Ala Asn Pro Ser Phe Asn Val Ile Ser Thr Gly His Ser Leu Gly
                165                 170                 175

Gly Ala Val Ala Val Leu Ala Ala Ala Asn Leu Arg Val Gly Gly Thr
            180                 185                 190

Pro Val Asp Ile Tyr Thr Tyr Gly Ser Pro Arg Val Gly Asn Ala Gln
        195                 200                 205

Leu Ser Ala Phe Val Ser Asn Gln Ala Gly Gly Glu Tyr Arg Val Thr
    210                 215                 220

His Ala Asp Asp Pro Val Pro Arg Leu Pro Pro Leu Ile Phe Gly Tyr
225                 230                 235                 240

Arg His Thr Thr Pro Glu Phe Trp Leu Ser Gly Gly Gly Gly Asp Lys
                245                 250                 255

Val Asp Tyr Thr Ile Ser Asp Val Lys Val Cys Glu Gly Ala Ala Asn
            260                 265                 270

Leu Gly Cys Asn Gly Gly Thr Leu Gly Leu Asp Ile Ala Ala His Leu
        275                 280                 285

His Tyr Phe Gln Ala Thr Asp Ala Cys Asn Ala Gly Gly Phe Ser Trp
    290                 295                 300

Arg Arg Tyr Arg Ser Ala Glu Ser Val Asp Lys Arg Ala Thr Met Thr
305                 310                 315                 320

Asp Ala Glu Leu Glu Lys Lys Leu Asn Ser Tyr Val Gln Met Asp Lys
                325                 330                 335

Glu Tyr Val Lys Asn Asn Gln Ala Arg Ser *
            340                 345
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CLAIMS

1. A process for reducing the content of phosphorus containing components in an edible oil, having from 50 to 10.000 part per million (ppm) of phosphorus content, which method comprises contacting said oil at a pH from 1.5 to 8 with an aqueous solution of a phospholipase A1 (PLA1), phospholipase A2 (PLA2), or phospholipase B (PLB) which is emulsified in the oil until the phosphorus content of the oil is reduced to less than 12 ppm, and then separating the aqueous phase from the treated oil,
and wherein said process is characterized by that said emulsified condition is formed using from 0.01 to 1.5 percent of water by weight of the oil, preferably from 0.01 to 1.0 percent of water by weight of the oil, and most preferably from 0.01 to 0.5 percent of water by weight of the oil.

2. The process according to claim 1, wherein said oil is an oil from which mucilage has previously been removed and which has a phosphorus content from 50 to 250 ppm.

3. The process according to claims 1 or 2, wherein the phospholipase is an phospholipase obtained from a microorganism, preferably a filamentous fungus, a yeast, or a bacterium.

4. The process according to claim 3, wherein the filamentous fungus is a species within the genus *Fusarium*, such as a strain of *F. culmorum*, F. *heterosporum*, *F. solani*, or in particular a strain of *F. oxysporum*.

5. The process according to claim 3, wherein the filamentous fungus is a species within the genus *Aspergillus*, such as a strain of *Aspergillus awamori*, *Aspergillus foetidus*, *Aspergillus japonicus*, *Aspergillus niger* or in particular *Aspergillus oryzae*.

6. The process according to any of the preceeding claims, wherein the phospholipase is a phospholipase which is

substantively independent of Ca^{2+} concentration measured as,

relative phospholipase activity at 5 mM EDTA and 5mM Ca^{2+} in a phospholipase activity assay measuring release of free fatty acids from lecithin in a buffer comprising 2% lecithin, 2% Triton X-100, 20 mM citrate, pH 5; incubated for 10 min. at 37°C followed by stop of reaction at 95°C for 5 min.;

wherein the ratio of relative phospholipase activity at 5mM EDTA/5 mM Ca^{2+} is greater than 0.25, more preferably greater than 0.5.

7. The process according to any of the preceding claims, wherein the phospholipase is a phospholipase which has a phospholipase activity which is capable of releasing at least 7 µmol of free fatty acid/min./mg enzyme; more preferably at least 15 µmol of free fatty acid/min./mg enzyme; measured as,

phospholipase activity is measured in an assay measuring release of free fatty acids from lecithin in a buffer comprising 2% lecithin, 2% Triton X-100, 20 mM citrate, pH 5; incubated for 10 min. at 37°C followed by stop of reaction at 95°C for 5 min..

8. The process according to any of the preceding claims, wherein the phospholipase is a phospholipase having an polypeptide sequence selected from the group comprising of:

(a) polypeptide having an amino acid sequence as shown in positions 31-346 of SEQ ID NO 1;

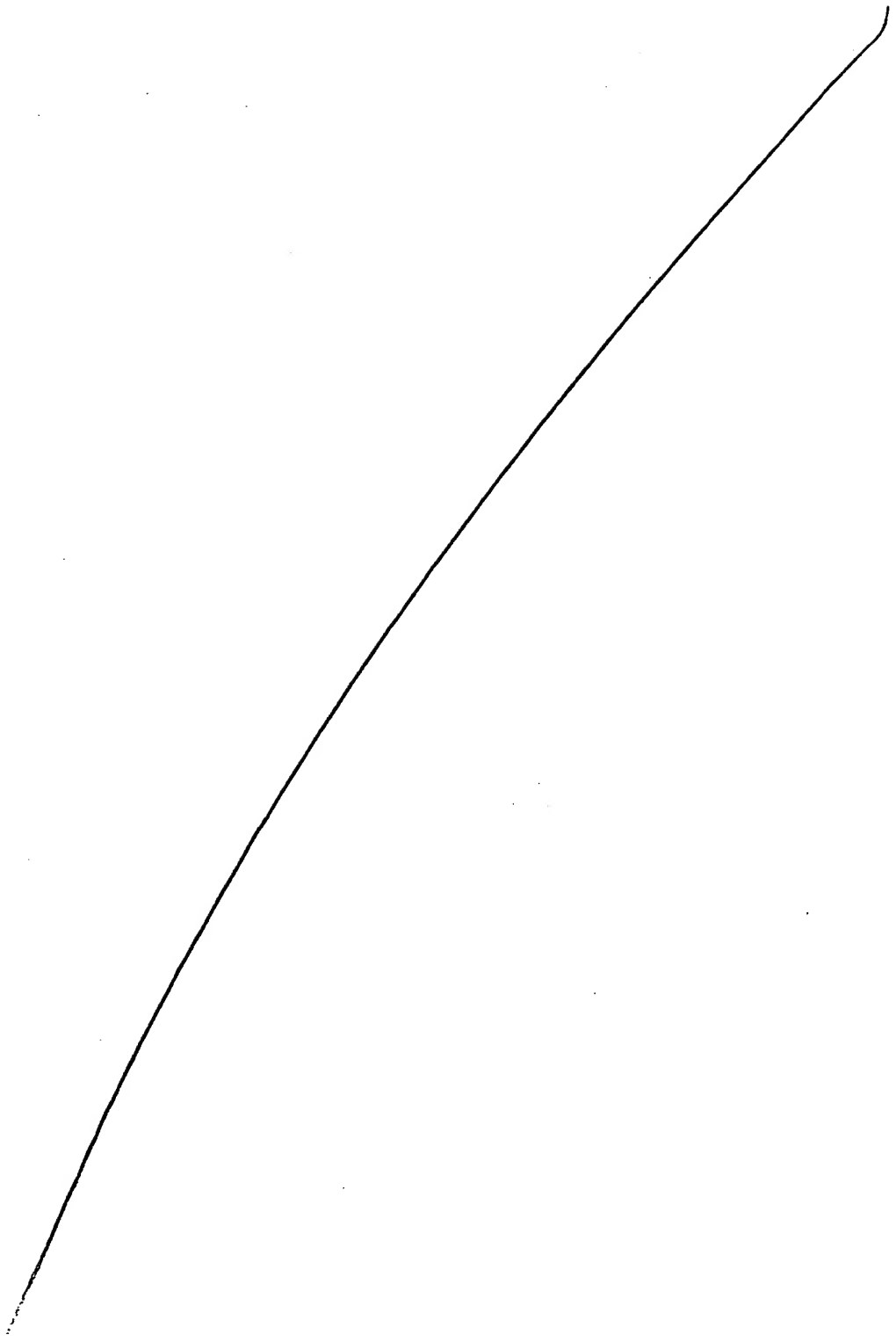
(b) a polypeptide having an amino acid sequence as shown in position 31-303 of SEQ ID NO 1;

(c) a polypeptide which is at least 70 % homologous with said polypeptide defined in (a), or (b); and

a fragment of (a), (b) or (c).

ABSTRACT

An improved process for enzymatic reducing the content of phosphorus containing components in an edible oil.



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Zeichen/Ref./Réf.	Anmeldung Nr./Application No./Demande n° /Patent Nr /Patent No./Brevet n°.
	99911648.6- -PCT/DK9900202
Anmelder/Applicant/Demandeur/Patentinhaber/Proprietor/Titulaire	
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NOTE: The following information concerns the steps which you are required to take for entry into the regional phase before the EPO. You are strongly advised to read it carefully. Failure to take the appropriate steps in due time could lead to the application being deemed withdrawn.

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C11B 3/00 — A1

(11) International Publication Number: **WO 99/53001**

(43) International Publication Date: 21 October 1999 (21.10.99)

(21) International Application Number: PCT/DK99/00202

(22) International Filing Date: 7 April 1999 (07.04.99)

(30) Priority Data:
0506/98 8 April 1998 (08.04.98) DK

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(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: AN ENZYMATIC OIL-DEGUMMING PROCESS

(57) Abstract

An improved process for enzymatic reducing the content of phosphorus containing components in an edible oil. The method comprises the use of phospholipase and a low amount of water.

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TITLE:

An enzymatic oil-degumming process

FIELD OF INVENTION

The present invention relates to an improved process for enzymatic reducing the content of phosphorus containing components in an edible oil.

BACKGROUND OF THE INVENTION

Oils obtained from the usual oil and fat production processes by compressing oil-bearing materials or by extracting oil from the materials and removing the extraction solvent contain impurities such as polar lipids mainly composed of phospholipids, as well as fatty acids, pigments, odor components and the like. Thus it is necessary to remove these impurities by a refining process. Such a process may require a degumming step.

In the art it is known to use phospholipase for enzymatic degumming of an edible oil (US 5,264,367; JP-A-2153997; and EP 622446), to reduce the phosphorus content of said water degummed edible oil.

However those references do not specifically suggest to use low amount of water in the enzymatic degumming process.

In contrary EP 622446 suggest to use high amount of water in the enzymatic degumming process. See page 3, line 33-44 and claim 4 in said document, which suggest to use more than 30 percent of water by weight of the oil in said process.

SUMMARY OF THE INVENTION

The problem, to be solved, by the present invention is to provide a simplified and economically cheaper process for enzymatic degumming of edible oils.

The solution is to perform said process using low amounts of water.

Accordingly, the present invention relates to a process for reducing the content of phosphorus containing components in an edible oil, having from 50 to 10.000 part per million (ppm) of phosphorous content, which method comprises contacting said oil at a pH from 1.5 to 8 with an aqueous solution of a

phospholipase A1 (PLA1), phospholipase A2 (PLA2), or phospholipase B (PLB) which is emulsified in the oil until the phosphorous content of the oil is reduced to less than 12 ppm, and then separating the aqueous phase from the treated oil, and wherein said process is characterized by that said emulsified condition is formed using from 0.01 to 1.5 percent of water by weight of the oil, preferably from 0.01 to 1.0 percent of water by weight of the oil, more preferably from 0.01 to 0.75 percent of water by weight of the oil, even more preferably from 0.01 to 0.5 percent of water by weight of the oil, and most preferably from 0.01 to 0.4 percent of water by weight of the oil.

Further, the lower range above of 0.01 percent of water by weight of the oil, may preferably be 0.1 percent of water by weight of the oil.

An advantage of the process described herein is that costs for water and waste water treatment may be reduced. Furthermore, oil recovery yields may be increased because less amount of oil will be wasted to the aqueous phase.

Further, an advantage of the process described herein may be that an oil-mill using this process may skip sludge recycling of the polluted water used in the process.

The in the art known enzymatic degumming processes give rise to a high amount of polluted water, which is expensive to clean up. This is of course an economically burden.

Further oil-mills traditionally have been forced to implement recycling of the water processes in order to save cost in said purifying of the polluted water.

Said recycling step may be saved by the low amount of water used in the process described herein.

In enzymatic degumming carried out according to the art (e.g. US 5,264,367) a heat treatment to e.g. 65-75 °C of the water in oil emulsion is usually carried out in order to facilitate separation of the oil and aqueous phases by e.g. centrifugation. When using the thermostable phospholipase Lecitase™ (Novo Nordisk A/S, Denmark) in the oil degumming process, the aqueous phase containing the enzyme can advantageously be reused several times (with or without addition

of fresh enzyme solution).

However, for the oil mill it may be advantageous if the recycling of the aqueous phase could be totally omitted. This would in the normal case mean that overall water consumption would be increased with increased costs. If only a low amount of water is used in the enzymatic degumming process, recycling of the sometimes problematic sludge phase could be omitted.

Embodiment(s) of the present invention is described below, by way of example(s) only.

DETAILED DESCRIPTION OF THE INVENTION

Edible oils:

In principle any edible oil may be degummed according to a process of the invention. Example of oils are crude oils and water degummed oils.

A crude oil (also called a non-degummed oil) may be a pressed or extracted oil or a mixture thereof from e.g. rapeseed, soybean, or sunflower. The phosphatide content in a crude oil may vary from 0.5-3% w/w corresponding to a phosphorus content in the range of 200-10.000 ppm, more preferably in the range of 250-1200 ppm. Apart from the phosphatides the crude oil also contains small concentrations of carbohydrates, sugar compounds and metal/phosphatide acid complexes of Ca, Mg and Fe.

Preferably, said edible oil is an oil from which mucilage has previously been removed and which has a phosphorus content from 50 to 250 ppm.

Such an oil is generally obtained by a water-degumming process and termed "a water-degummed oil".

A water-degummed oil is typically obtained by mixing 1-3% w/w of hot water with warm (60-90°C) crude oil. Usual treatment periods are 30-60 minutes. The water-degumming step removes the phosphatides and mucilaginous gums which become insoluble in the oil when hydrated. The hydrated phosphatides and gums can be separated from the oil by settling, filtering or centrifuging - centrifuging being the more prevalent practice.

Alternatively, the process here termed "water-degumming" may be called "wet refining to remove mucilage" (see US 5,264,367).

Further, an edible is preferably an vegetable oil.

A Phospholipase used in the process:

Preferably, a phospholipase used in the process of the invention is a phospholipase obtained from a microorganism, preferably a filamentous fungus, a yeast, or a bacterium.

For the purpose of the present invention the term "obtained from", as used herein in connection with a specific microbial source, means that the enzyme and consequently the DNA sequence encoding said enzyme is produced by the specific source.

The enzyme is then obtained from said specific source by standard known methods enabling the skilled person to obtain a sample comprising the enzyme and capable of being used in a process of the invention. Said standard methods may be direct purification from said specific source or cloning of a DNA sequence encoding the enzyme followed by recombinant expression either in the same source (homologous recombinant expression) or in a different source (heterologous recombinant expression).

More preferably, a phospholipase used in a process of the invention is obtained from a filamentous fungal species within the genus *Fusarium*, such as a strain of *F. culmorum*, F. *heterosporum*, *F. solani*, or in particular a strain of *F. oxysporum*; or

a filamentous fungal species within the genus *Aspergillus*, such as a strain of *Aspergillus awamori*, *Aspergillus foetidus*, *Aspergillus japonicus*, *Aspergillus niger* or in particular *Aspergillus oryzae*.

Examples of suitable *Fusarium* phospholipases are disclosed in

i) Tsung-Che et al. (Phytopathological notes 58:1437-38 (1968)) (a phospholipase from *Fusarium solani)*; and

ii) EP Patent Application No. 97610056.0 disclosing a suitable *F. culmorum PL* (see example 18 in said doc.) and a suitable *F. oxysporum PL* (see example 1-17).

Suitable *Aspergillus* phospholipases are diclosed in

i) EP 575133 disclosing numerous different Aspergillus PL`s (see claim 14) and in particular a PL from A. oryzae(Claim

17 or 18) and a PL from A. niger (claim 19); and

ii) DE 19527274 A1 dicloses a suitable Aspergillus preparation (see examples).

Further the commercial available phospholipase preparation Degomma VOD (Roehm, Germany), which is believed to comprise an Aspergillus phospholipase is suitable to be used in a process of the invention.

Further, it is preferred that a phospholipase used in a process of the invention exhibits certain properties.

Accordingly, embodiment of the invention relates to

i) a process according to the invention, wherein the phospholipase is a phospholipase which is substantively independent of Ca^{2+} concentration measured as,

relative phospholipase activity at 5 mM EDTA and 5mM Ca^{2+} in a phospholipase activity assay measuring release of free fatty acids from lecithin in a buffer comprising 2% lecithin, 2% Triton X-100, 20 mM citrate, pH 5; incubated for 10 min. at 37°C followed by stop of reaction at 95°C for 5 min.;
wherein the ratio of relative phospholipase activity at 5mM EDTA/5 mM Ca^{2+} is greater than 0.25, more preferably greater than 0.5; and/or

ii) a process according to the invention, wherein the phospholipase is a phospholipase which has a phospholipase activity which is capable of releasing at least 7 µmol of free fatty acid/min./mg enzyme; more preferably at least 15 µmol of free fatty acid/min./mg enzyme; measured as,

phospholipase activity is measured in an assay measuring release of free fatty acids from lecithin in a buffer comprising 2% lecithin, 2% Triton X-100, 20 mM citrate, pH 5; incubated for 10 min. at 37°C followed by stop of reaction at 95°C for 5 min..

A detailed description of above mentioned assays is disclosed in a working example herein (*vide infra*). For even further details reference is made to EP Patent Application No. 97610056.0 (see example 9 in said document).

Further it has been demonstrated that a phospholipase special suited for enzymatic oil degumming in general and in

particular for the improved process described herein is characterized by having a certain primary amino acid sequence.

Accordingly, in an even further embodiment the invention relates to a process according to the invention, wherein the phospholipase is a phospholipase having an polypeptide sequence selected from the group comprising of:

(a) polypeptide having an amino acid sequence as shown in positions 31-346 of SEQ ID NO 1;

(b) a polypeptide having an amino acid sequence as shown in position 31-303 of SEQ ID NO 1;

(c) a polypeptide which is at least 70 % homologous with said polypeptide defined in (a), or (b); and

a fragment of (a), (b) or (c).

For a detailed description of cloning and purification of a phospholipase having the above mentioned polypeptide sequence reference is made to EP Patent Application No. 97610056.0.

In this document it can further be seen that a phospholipase obtained from *F. oxysporum* and having the polypeptide sequence shown in (b) above exhibits both of the above mentioned functional characteristic. Accordingly, this phospholipase is the most preferred phospholipase to be used in a process of the invention. A working example herein demonstrates the use of this phospholipase (vide infra).

Finally an example of a suitable non-microbial phospholipase is the commercial available PL (Lecitase™, Novo Nordisk A/S, Denmark) obtained from porcine pancreas.

<u>Standard process parameters of the process of the invention:</u>

Besides the specific use of low amount of water in the process of the invention, any of the other process parameters may be done according to the art. See Background section above for references to the art known processes.

The enzymatic treatment is conducted by dispersing an aqueous solution of the phospholipase, preferably as droplets with an average diameter below 10 μ(micro)m.

According to the process of the invention the amount of water is from 0.01 to 1.5% by weight in relation to the oil.

An emulsifier may optionally be added. Mechanical agitation may be applied to maintain the emulsion.

The enzymatic treatment can be conducted at any pH in the range 1.5-8, preferably from pH 3-6. The pH may be adjusted by adding citric acid, a citrate buffer, NaOH or HCl.

A suitable temperature is generally 30-75°C (particularly 40-60°C). The reaction time will typically be 0.5-12 hours (e.g. 2-6 hours), and a suitable enzyme dosage will usually be 100-5000 IU per liter of oil, particularly 200-2000 IU/l.

The enzymatic treatment may be conducted batchwise, e.g. in a tank with stirring, or it may be continuous, e.g. a series of stirred tank reactors.

The enzymatic treatment is followed by separation of an aqueous phase and an oil phase. This separation may be performed by conventional means, e.g. centrifugation. The process of the invention can reduce this value to below 12 ppm, more preferably below 10, and even more preferably below 5 ppm.

MATERIALS AND METHODS

EXAMPLES

EXAMPLE 1

General description of assay for enzymatic degumming of edible oil

Equipment for carrying out enzymatic degumming

The equipment consists of a 1 l jacketed steel reactor fitted with a steel lid, a propeller (about 600 rpm), baffles, a temperature sensor, an inlet tube at the top, a reflux condenser (about 4°C) at the top, and an outlet tube at the bottom. The reactor jacket is connected to a thermostat bath. The outlet tube is connected via silicone tubing to a Silverson in-line mixer head equipped with a "square hole high shear screen", driven by a Silverson L4RT high shear lab mixer (about 8500 rpm, flow ca. 1.1 l/minute). The mixer head is fitted with a cooling coil (5-10 °C) and an outlet tube, which is connected to the

inlet tube of the reactor via silicone tubing. A temperature sensor is inserted in the silicone tubing just after the mixer head. The only connection from the reactor/mixer head system to the atmosphere is through the reflux condenser.

General procedure for carrying out enzymatic degumming

All cooling and thermostat equipment is turned on. Then 0.6 l (ca. 560 g) of oil is loaded in the reactor, which is kept at about the temperature needed for the specific experiment. The lab mixer is turned on, whereby the oil starts to circulate from the reactor to the mixer head and back to the reactor. The system is allowed to equilibrate for about 10 minutes, during which period the temperature is fine tuned. The pre-treatment period starts with addition of 0.6 g (2.86 mmol) citric acid monohydrate in the appropriate amount of water or the appropriate amount of a mixture of citric acid and trisodium citrate (see Tables 1 and 7 below; [citric acid] in water/oil emulsion = 4.6 mM), which sets t = 0. At t = 30 minutes the appropriate amount of 4 M NaOH solution is added (see Tables 1 and 7).

Table 1. Water content in Experiments A-D; wdg rape seed oil.

Experiment	Water content	Water in 560 g oil	Water added at t=0	Water in NaOH solution	Water in enzyme solution	Total water
A	5.3 %	0.56 g	27 g	1.1 g	1.0 g	29.7 g
B	1.3 %	0.56 g	5.0 g	0.7 g	1.0 g	7.3 g
C	0.3 %	0.56 g	0.05 g*	0 g	1.0 g	1.6 g
D	0.3 %	0.56 g	0.07 g**	0 g	1.0 g	1.6 g

* Water contribution from o.6 g citric acid monohydrate.

** Water contribution from mixt. of 0.5 g citric acid monohydrate and 0.14 g trisodium citrate dihydrate.

At t = 35 minutes samples are drawn for P-analysis and pH determination. Just after this the required amount of enzyme

solution is added (end of pre-treatment period). Samples for P-analysis and pH determination are drawn at t = 1, 2, 3.5, 5, 6 hours, and then the reaction is stopped.

The reactor/mixer system is emptied and rinsed with 2x500 ml 10% Deconex/DI water solution followed by minimum 3x500 ml of DI water. Table 2 is a presentation of the various additions and samplings during the reaction.

Table 2. Schedule for enzymatic degumming

Time	Addition of	Sampling	
		P-analysis	pH determination
		X	
0	Citric acid		
5 min.			X
30 min.		X	X
30 + δ min.	NaOH		
35 min.		X	X
35 + δ min.	Enzyme		
1 hour		X	X
2 hours		X	X
3.5 hours		X	X
5 hours		X	X
6 hours		X	X

Phosphorus analysis:

Sampling for P-analysis:

Take 10 ml of water in oil emulsion in a glass centrifuge tube. Heat the emulsion in a boiling water bath for 30 minutes. Centrifuge at 5000 rpm for 10 minutes. Transfer about 8 ml of upper (oil) phase to a 12 ml polystyrene tube and leave it (to settle) for 12-24 hours. After settling draw about 1-2 g from the upper clear phase for P-analysis.

P-analysis was carried out according to procedure 2.421 in

"Standard Methods for the Analysis of Oils, Fats, and Derivatives, 7th ed. (1987)":

Weigh 100 mg of MgO (leicht, Merck #5862) in a porcelain dish and heat with a gas burner. Add 1-2 g of oil and ignite with a gas burner to give a black, hard mass. Heat in a Vecstar furnace at 850°C for 2 hours to give white ashes. Dissolve the ashes in 5 ml of 6 M HNO_3 and add 20 ml of reagent mix. Leave for 20 minutes. Measure absorbance at 460 nm (use a blank (5 ml HNO_3 + 20 ml reagent mix) for zero adjustment). Calculate by using calibration curve.

pH determination

Take 2 ml of water in oil emulsion and mix with 2 ml of MilliQ water. After phase separation, pipette off top oil layer. Measure pH in aqueous phase with pH electrode Orion. Measurements are transformed to "real" pH values by the formula

$$pH_{real} = pH_{measured} - 0.38.$$

A calibration curve was obtained by dissolving 0.6 g of citric acid monohydrate in 27 g of DI water; pH of this solution was measured by pH electrode Orion (pH_{real}). 100 µl were mixed with 2 ml MilliQ water, and pH of this solution was measured by pH electrode Orion ($pH_{measured}$). pH of the citric acid solution was changed gradually by adding NaOH solution, and for each adjustment dilution and pH measurements were carried out as described above.)

EXAMPLE 2

Degumming of water-degummed rape seed oil (I)

Experiments were carried out according to the "General procedure for carrying out enzymatic degumming" as described in example 1 above.

Oil:

Water-degummed rape seed oil from Århus Oliefabrik (AOM), Denmark. Batches C00730/B01700 and C00730/B01702, P-content 231-236 ppm. Water content ≤ 0.1 % w/w.

Enzyme:

PL from *Fusarium oxysporum* having the amino acid sequence shown in SEQ NO 1.

Batch F-9702027, estimated conc. 0.75 mg/ml.

The enzyme was recombinantly expressed and purified as described in EP Patent application number 97610056.0.

Experiment A (water content 5.3 %)

0.6 l (560 g) of wdg rape seed oil is loaded in the equipment and heated to 40°C. At t = 0 min. a solution of 0.6 g of citric acid monohydrate in 27 g of water was added. At t = 30 min. 1.07 ml (4.3 mmoles) of 4 M NaOH solution were added, which yield a pH of about 5. At t = 35 min., 1 ml (0.75 mg) of a purified solution of phospholipase from *F. oxysporum* is added. The measured phosphorus content in the oil phase after centrifugation as well as the pH values in the aqueous phase is shown in Table 3.

Table 3. Results from degumming of wdg rape seed oil with phospholipase from *F. oxysporum*, water content 5.3 %.

Time (hours)	Phosphorus content in oil phase	pH
0	243	
0.50	215	4.7
0.58	216	5.5
1.0	66	4.9
2.0	10	4.9
3.5	8	5.4
5.0	9	5.0

Experiment B (water content 1.3 %)

As in Experiment A above except that at t = 0 min. 0.6 g of

citric acid monohydrate in 5.0 g of water was added, and at t = 30 min. 0.71 ml (2.86 mmoles) of 4 M NaOH solution were added which yield a pH of about 5. The measured phosphorus content in the oil phase after centrifugation as well as the pH values in the aqueous phase is shown in Table 4.

Table 4. Results from degumming of wdg rape seed oil with phospholipase from *F. oxysporum*, water content 1.3 %.

Time (hours)	Phosphorus content in oil phase	pH
0	237	
0.50	213	4.7
0.58	197	5.7
1.0	78	4.9
2.0	9	4.9
3.5	10	5.0
5.0	12	5.1
6.0	10	5.0

Experiment C (water content 0.3 %)

As in Experiment A above except that at t = 0 min. 0.6 g of citric acid monohydrate powder was added, and at t = 30 min. no NaOH solution was added, which yield a pH of about 5. The measured phosphorus content in the oil phase after centrifugation as well as the pH values in the aqueous phase is shown in Table 5.

Table 5. Results from degumming of wdg rape seed oil with phospholipase from *F. oxysporum*, water content 0.3 %.

Time (hours)	Phosphorus content in oil phase	pH
0	246	4.9
0.50	234	5.1
0.58		
1.0	101	4.8
2.0	18	5.2
3.5	11	5.2

Experiment D (water content 0.3 %)

As in Experiment C above except that at t = 0 min. a mixture of 0.5 g of citric acid monohydrate and 0.14 g trisodium citrate dihydrate powder was added, which yield a pH of about 5. The measured phosphorus content in the oil phase after centrifugation as well as the pH values in the aqueous phase is shown in Table 6.

Table 6. Results from degumming of wdg rape seed oil with phospholipase from *F. oxysporum*, water content 0.3 %.

Time (hours)	Phosphorus content in oil phase	pH
0	243	
0.50	244	5.5
0.58		
1.0	101	5.1
2.0	8	4.9

EXAMPLE 3

Degumming of crude (mixture of pressed and extracted) rape seed oil (II)

Experiments were carried out according to the "General procedure for carrying out enzymatic degumming" as described in example 1 above.

Oil:

Crude rape seed oil from MILO Olomouk, Czech rep. Batch C00745/B02042, P-content 263 ppm. Water content 0.17 % w/w.

Table 7. Water content in Experiments E and F; crude rape seed oil.

Experiment	Water content	Water in 560 g oil	Water added at t=0	Water in NaOH solution	Water in enzyme solution	Total water
E	5.4 %	0.95 g	27 g	1.1 g	1.0 g	30.1 g
F	1.4 %	0.95 g	5.0 g	0.7 g	1.0 g	7.7 g

Experiment E (water content 5.4 %)

0.6 l (560 g) of crude rape seed oil is loaded in the equipment and heated to 40°C. At t = 0 min. a solution of 0.6 g of citric acid monohydrate in 27 g of water was added. At t = 30 min. 1.07 ml (4.3 mmoles) of 4 M NaOH solution were added, which yield a pH of about 5. At t = 35 min., 1 ml (0.75 mg) of a purified solution of phospholipase from *F. oxysporum* is added. The measured phosphorus content in the oil phase after centrifugation as well as the pH values in the aqueous phase is shown in Table 8.

Table 8. Results from degumming of crude rape seed oil with phospholipase from *F. oxysporum*, water content 5.4 %.

Time (hours)	Phosphorus content in oil phase	pH
0	222	
0.50	165	
0.58	136	4.8
1.0	38	5.1
2.0	10	5.0
3.5	11	5.0
5.0	11	5.0
6.0	10	5.3

Experiment F (water content 1.4 %)

As in Experiment E above except that at t = 0 min. 0.6 g of citric acid monohydrate in 5.0 g of water was added, and at t = 30 min. 0.71 ml (2.86 mmoles) of 4 M NaOH solution were added which yield a pH of about 5. The measured phosphorus content in the oil phase after centrifugation as well as the pH values in the aqueous phase is shown in Table 9.

Table 9. Results from degumming of crude rape seed oil with phospholipase from *F. oxysporum*, water content 1.4 %.

Time (hours)	Phosphorus content in oil phase	pH
0	223	
0.50	119	
0.58	92	5.1
1.0	31	5.1
2.0	12	5.0
3.5	11	5.1
5.0	9	4.8
6.0	8	4.3

EXAMPLE 4

Assays used for characterization of a phospholipase suitable to be used in an oil degumming process of the invention.

Phospholipase activity assays:

Phospholipase activity (PHLU) was measured as the release of free fatty acids from lecithin. 50 µl 4% L-alpha-phosphatidylcholine (plant lecithin from Avanti, USA), 4% Triton X-100, 5 mM $CaCl_2$ in 50 mM HEPES, pH 7 was added, 50 µl enzyme solution diluted to an appropriate concentration in 50 mM HEPES, pH 7. The samples were incubated for 10 min at 30°C and the reaction stopped at 95°C for 5 min prior to centrifugation (5 min at 7000 rpm). Free fatty acids were determined using the NEFA C kit from Wako Chemicals GmbH; 25 µl reaction mixture was added to 250 µl reagent A and incubated for 10 min at 37°C. Then 500 µl Reagent B was added and the sample was incubated again, 10 min at 37°C. The absorption at 550 nm was measured using an HP 8452A diode array spectrophotometer. Samples were run at least in duplicates. Substrate and enzyme blinds (preheated enzyme samples (10 min at 95°C) + substrate) were included. Oleic acid was used as a fatty acid standard. 1 PHLU equals the amount of enzyme capable of releasing 1 µmol of free fatty acid/min under these conditions.

Alternatively, the assay was run at 37°C in 20 mM citrate buffer, pH 5 (Ca^{2+}-dependence) or 20 mM Britton-Robinson buffer (pH-profile/temperature-profile/stability).

Phospholipase A1 activity (PLA1) was measured using 1-(S-decanoyl)-2-decanoyl-1-thio-sn-glycero-3-phosphocholine (D3761 Molecular Probes) as a substrate. 190 µl substrate (100 µl D3761 (2 mg/ml in ethanol) + 50 µl 1 % Triton X-100 + 1.85 ml 50 mM HEPES, 0.3 mM DTNB, 2 mM $CaCl_2$, pH 7) in a 200 µl cuvette were added to 10 µl enzyme, and the absorption at 410 nm was measured as a function of time on the HP 8452A diode array spectrophotometer at room temperature. Activity was calculated as the slope of the curve in the linear range. PLA1 equals the amount of enzyme capable of releasing 1 µmol of free fatty acid (thiol)/min at these conditions.

Phospholipase A2 activity (PLA2) was measured at 40°C using 1-hexadecanoyl-2-(1-pyrenedecanoyl)-sn-glycero-3-phosphocholine (H361 Molecular Probes). 2 ml substrate (50 µl 1% Triton X-100 + 25 µl 0.1% H361 in methanol + 10 ml 50mM HEPES, pH 7) in a 2 ml cuvette with stirring was added to 10 µl enzyme, and the pyrene fluorescence emission was measured at 376 nm (excitation at 340 nm) as a function of time (1 sec. intervals) using the Perkin Elmer LS50 apparatus. In the Triton X-100/phospholipid micelles the concentration of phospholipid was adjusted to have excimer formation (emits at 480 nm). Upon cleavage the fatty acid in the 2-position containing the pyrene group is released into the aqueous phase resulting in an increase in the monomer emission. PLA2 was taken as the slope of the curve in the linear range at equal conditions.

CLAIMS

1. A process for reducing the content of phosphorus containing components in an edible oil, having from 50 to 10.000 part per million (ppm) of phosphorus content, which method comprises contacting said oil at a pH from 1.5 to 8 with an aqueous solution of a phospholipase A1 (PLA1), phospholipase A2 (PLA2), or phospholipase B (PLB) which is emulsified in the oil until the phosphorus content of the oil is reduced to less than 12 ppm, and then separating the aqueous phase from the treated oil, and wherein said process is characterized by that said emulsified condition is formed using from 0.01 to 1.5 percent of water by weight of the oil, preferably from 0.01 to 1.0 percent of water by weight of the oil, and most preferably from 0.01 to 0.5 percent of water by weight of the oil.

2. The process according to claim 1, wherein said oil is an oil from which mucilage has previously been removed and which has a phosphorus content from 50 to 250 ppm.

3. The process according to claims 1 or 2, wherein the phospholipase is an phospholipase obtained from a microorganism, preferably a filamentous fungus, a yeast, or a bacterium.

4. The process according to claim 3, wherein the filamentous fungus is a species within the genus *Fusarium*, such as a strain of *F. culmorum*, F. *heterosporum*, *F. solani*, or in particular a strain of *F. oxysporum*.

5. The process according to claim 3, wherein the filamentous fungus is a species within the genus *Aspergillus*, such as a strain of *Aspergillus awamori*, *Aspergillus foetidus*, *Aspergillus japonicus*, *Aspergillus niger* or in particular *Aspergillus oryzae*.

6. The process according to any of the preceeding claims, wherein the phospholipase is a phospholipase which is substantively independent of Ca^{2+} concentration measured as, relative phospholipase activity at 5 mM EDTA and 5mM Ca^{2+} in a phospholipase activity assay measuring release of free fatty

acids from lecithin in a buffer comprising 2% lecithin, 2% Triton X-100, 20 mM citrate, pH 5; incubated for 10 min. at 37°C followed by stop of reaction at 95°C for 5 min.;
wherein the ratio of relative phospholipase activity at 5mM EDTA/5 mM Ca^{2+} is greater than 0.25, more preferably greater than 0.5.

7. The process according to any of the preceding claims, wherein the phospholipase is a phospholipase which has a phospholipase activity which is capable of releasing at least 7 µmol of free fatty acid/min./mg enzyme; more preferably at least 15 µmol of free fatty acid/min./mg enzyme; measured as,
phospholipase activity is measured in an assay measuring release of free fatty acids from lecithin in a buffer comprising 2% lecithin, 2% Triton X-100, 20 mM citrate, pH 5; incubated for 10 min. at 37°C followed by stop of reaction at 95°C for 5 min..

8. The process according to any of the preceding claims, wherein the phospholipase is a phospholipase having an polypeptide sequence selected from the group comprising of:
(a) polypeptide having an amino acid sequence as shown in positions 31-346 of SEQ ID NO 1;
(b) a polypeptide having an amino acid sequence as shown in position 31-303 of SEQ ID NO 1;
(c) a polypeptide which is at least 70 % homologous with said polypeptide defined in (a), or (b); and
a fragment of (a), (b) or (c).

SEQUENCE LISTING

<110> NOVO NORDISK A/S

<120> AN ENZYMATIC OIL-DEGUMMING PROCESS

<130> 5570-WO

<140>
<141>

<160> 1

<170> PatentIn Ver. 2.0

<210> 1
<211> 346
<212> PRT
<213> Fusarium oxysporum

<400> 1

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         35                  40                  45

Gly Ala Ala Ala Tyr Cys Asn Ser Glu Ala Ala Ala Gly Ser Lys Ile
     50                  55                  60

Thr Cys Ser Asn Asn Gly Cys Pro Thr Val Gln Gly Asn Gly Ala Thr
 65                  70                  75                  80

Ile Val Thr Ser Phe Val Gly Ser Lys Thr Gly Ile Gly Gly Tyr Val
                 85                  90                  95

Ala Thr Asp Ser Ala Arg Lys Glu Ile Val Val Ser Phe Arg Gly Ser
            100                 105                 110

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Pro Val Asp Ile Tyr Thr Tyr Gly Ser Pro Arg Val Gly Asn Ala Gln
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            260                 265                 270

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 99/00202

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C11B 3/00
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C11B

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO 9826057 A1 (NOVO NORDISK A/S), 18 June 1998 (18.06.98), See sequence page 17, line 14-15 --	1-8
P,X	WO 9818912 A1 (NOVO NORDISK A/S), 7 May 1998 (07.05.98), See page 8, line 25, claim 27 --	1-8
X	File WPI, Derwent accession no. 90-226962, Showa Sangyo Co: "Purificn. of fat and oil, requiring no acid-removing process - by treating with enzyme having phospho-lipase A activity", JP,A,2153997, 900613, DW9030 --	1-8

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" erlier document but published on or after the international filing date
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"O" document referring to an oral disclosure, use, exhibition or other means
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"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
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Date of the actual completion of the international search	Date of mailing of the international search report
1 July 1999	17 -07- 1999
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Yvonne Siösteen/Els Telephone No. +46 8 782 25 00

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 99/00202

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	File WPI, Derwent accession no. 90-096521, Showa Sangyo Co: "Lysolecithin prepn. - by adding enzyme showing phospholipase A activity to oil", JP,A,2049593, 900219, DW9013	1-8
	--	
X	US 5264367 A (ERIK AALRUST ET AL), 23 November 1993 (23.11.93), See column 3, line 3	1-8
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A	EP 0622446 A2 (SHOWA SANGYO CO., LTD.), 2 November 1994 (02.11.94), See page 3, lines 33-34, claim 4	1-8
	--	
A	US 5558781 A (HENNING BUCHOLD ET AL), 24 Sept 1996 (24.09.96)	1-8
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INTERNATIONAL SEARCH REPORT

Information on patent family members

01/06/99

International application No.
PCT/DK 99/00202

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		EP 0869167 A	07/10/98
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US 5264367 A	23/11/93	AT 120482 T	15/04/95
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		EP 0513709 A,B	19/11/92
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		ES 2072043 T	01/07/95
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		RU 2033422 C	20/04/95
EP 0622446 A2	02/11/94	DE 69408891 D,T	22/10/98
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		CA 2122069 A	26/10/94
US 5558781 A	24/09/96	AT 162210 T	15/01/98
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		CA 2136050 A	20/05/95
		CN 1112156 A	22/11/95
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		DE 59405028 D	00/00/00
		DK 654527 T	16/03/98
		EP 0654527 A,B	24/05/95
		SE 0654527 T3	
		ES 2111841 T	16/03/98
		GR 3026501 T	31/07/98
		JP 7188691 A	25/07/95

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Eingangsstelle	Receiving Section	Section de Dépôt

Novozymes A/S
Krogshoejvej 36
2880 Bagsvaerd
DANEMARK

Datum/Date
20/12/00

Zeichen/Ref./Réf.	Anmeldung Nr./Application No./Demande n°./Patent Nr./Patent No./Brevet n°.
5570.205-EP,SLK	99911648.6-2109 / 1071734
Anmelder/Applicant/Demandeur/Patentinhaber/Proprietor/Titulaire: Novozymes A/S	

NOTIFICATION OF EUROPEAN PUBLICATION NUMBER AND INFORMATION ON THE APPLICATION OF ARTICLE 67(3) EPC

The provisional protection under Article 67(1) and (2) EPC in the individual Contracting States becomes effective only when the conditions referred to in Article 67(3) EPC have been fulfilled (for further details, see information brochure of the European Patent Office "National Law relating to the EPC" and additional information in the Official Journal of the European Patent Office).

Pursuant to Article 158(1) EPC the publication under Article 21 PCT of an international application for which the European Patent Office is a designated Office takes the place of the publication of a European patent application.

The bibliographic data of the above-mentioned Euro-PCT application will be published on 31.01.01 in Section I.1 of the European Patent Bulletin.
The European publication number is 1071734.

In all future communications to the European Patent Office, please quote the application number plus Directorate number.

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Novozymes A/S
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2880 Bagsvaerd
DANEMARK

Datum/Date
11.12.00

Zeichen/Ref./Réf.	Anmeldung Nr./Application No./Demande n°./Patent Nr ./Patent No./Brevet n°.
5570.205-EP,SLK	99911648.6-2109/

Anmelder/Applicant/Demandeur/Patentinhaber/Proprietor/Titulaire
Novozymes A/S

C O M M U N I C A T I O N

concerning the registration of amendments relating to

[X] a transfer (Rule 20/Rules 61,20 EPC)

[ ] entries pertaining to the applicant/the proprietor (Rule 92(1)(f) EPC)

As requested, the entries pertaining to the applicant of the above-mentioned European patent application/to the proprietor of the above-mentioned European patent have been amended to the following:

DE-GB-NL
Novozymes A/S
Krogshoejvej 36
2880 Bagsvaerd/DK

The registration of the changes has taken effect on 25.11.00

In the case of a published application/a patent, the change will be recorded in the Register of European Patents and published in the European Patent Bulletin (Section I.12/II.12).

Your attention is drawn to the fact that, in the case of the registration of a transfer, any automatic debit order only ceases to be effective from the date of its express revocation (cf. point 14(c) of the Arrangements for the automatic debiting procedure, Supplement to OJ EPO 6/1994).

Formalities officer
Tel.: (+49-89) 2399-

Confirmation copy

novozymes®

European Patent Office
D-80298 München 2
Germany

17 November 2000

EPO - Munich
58
21. Nov. 2000

Dear Sirs

Re.: **Confirmatory assignment**
General authorization
Automatic debit order
Address for correspondence

Enclosed please find an Assignment confirming that

NOVO NORDISK A/S
Novo Allé
DK-2880 Bagsvaerd
Denmark

has assigned all its rights to the European patent applications listed in the enclosed Appendix I to

Novozymes A/S
Krogshoejvej 36
DK-2880 Bagsvaerd
Denmark.

When corresponding with us in the future in the European applications listed in Appendix I, please address all mail, including invoices and statements, to:

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Patents
Krogshoejvej 36
DK-2880 Bagsvaerd
Denmark

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Telephone:
+45 88 24 99 99
Telefax:
+45 44 42 60 80

Internet:
www.novozymes.com

CVR number:
10 00 71 27

M2 21.11.00

Also, we respectfully request that the Automatic Debit Order for the European applications listed in Appendix I continue to apply to our deposit account No. 2803.0007 in the name of Novozymes A/S. In this respect, we refer to our letter of 2 November 2000 for the attention of the Cash & Account, a copy of which we enclose.

Finally, we respectfully request that the enclosed General Authorization apply to the European patent applications listed in Appendix I.

Kind regards
Novozymes A/S

Gertrud Sonne Kofoed

M21.11.00

CONFIRMATORY ASSIGNMENT

THIS CONFIRMATORY ASSIGNMENT is made on the 17th day of November two thousand, BETWEEN Novo Nordisk A/S, a Danish company, of Novo Allé, DK-2880 Bagsvaerd, Denmark (hereinafter called "the Assignor") of the one part and Novozymes A/S, a Danish company, of Krogshoejvej 36, DK-2880 Bagsvaerd, Denmark (hereinafter called "the Assignee") of the other part.

WHEREAS:

A. The Assignor is registered owner of European Patent Applications as set out in the schedule appended (hereinafter referred to as "the Applications").

B. The parties hereto have transferred, for good and valuable consideration, the Assignor's rights in the Applications to the Assignee.

C. The parties hereto wish to confirm, for the purpose of recording the
D. transfer at the European Patent Office, that the rights in the Applications have been transferred to the Assignee.

NOW IT IS HEREBY AGREED THAT:

1. In consideration of the sum of one US dollar now paid by the Assignee to the Assignor (the receipt whereof is hereby acknowledged), the Assignor as registered owner confirms, by way of confirmatory assignment, that all its right, title and interest in and to the Applications (including any and all divisions, reissues, continuations and extensions thereof) are assigned to the Assignee free from all licences, charges or other encumbrances to the intent that a grant of European patents thereon shall be in the name of and shall vest in the Assignee TOGETHER WITH all the rights, powers, liberties and immunities arising or accrued therefrom including the right to sue for damages and other remedies in respect of any infringement of such rights or other rights within the scope of the

claims of any published specifications accompanying the Applications prior to the date hereof.

2. The Assignee hereby confirms that it accepts such assignment.

3. At the request and cost of the Assignee the Assignor will at all times hereafter assist the prosecution of the Applications to grant and will assist the defence of any proceedings by way of intervention or in opposition to the grant of the European patents pursuant to the Applications and will execute all such deeds and documents and do all such acts as may be necessary or desirable formally to register this Assignment at the European Patent Office and to render the Assignment effective under the national law of each Contracting State designated in the Applications and to procure the grant of European patents pursuant to the Applications.

4. The Assignee shall by virtue of this assignment be entitled to the grant direct to it in its own name of the European patents to be granted pursuant to the Applications.

SCHEDULE

Application No.	*Publ. No.*	*Our ref.*
92104421.0	489718	3061 212 EP
95107678.5	675196	3160 215 EP
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M 21.11.00

Signed for and on behalf of Novo Nordisk A/S

by

Mads Krogsgaard Thomsen	Kåre Schultz
Executive Vice President	Executive Vice President

Signed for and on behalf of Novozymes A/S

by

Per Falholt	Arne W. Schmidt
Executive Vice President	Executive Vice President

Witness...

1 **ALLGEMEINE VOLLMACHT**
GENERAL AUTHORISATION
POUVOIR GENERAL

Nur für amtlichen Gebrauch / For official use only
Cadre réservé à l'administration
Nr. der allgemeinen Vollmacht / General Authorisation No.
N° du pouvoir général

2 Ich (Wir) / I (We) / Je (Nous)

Novozymes A/S
Krogshøjvej 36
DK-2880 Bagsværd
DENMARK

3 bevollmächtige(n) hiermit / do hereby authorise / autorise (autorisons) par la présente

See attached additional sheet for a detailed list of representatives of Novozymes A/S

4 mich (uns) in den durch das Europäische Patentübereinkommen geschaffenen Verfahren in allen meinen (unseren) Patentangelegenheiten zu vertreten, alle Handlungen für mich (uns) vorzunehmen und Zahlungen für mich (uns) in Empfang zu nehmen.
to represent me (us) in all proceedings established by the European Patent Convention and to act for me (us) in all patent transactions and to receive payments on my (our) behalf.
à me (nous) représenter pour ce qui concerne toutes mes (nos) affaires de brevet dans toute procédure instituée par la Convention sur le brevet européen et, à ce titre, à agir en mon (notre) nom et à recevoir des paiements pour mon (notre) compte.

[X] Die Vollmacht gilt auch für Verfahren nach dem Vertrag über die internationale Zusammenarbeit auf dem Gebiet des Patentwesens.
This authorisation shall also apply to the same extent to any proceedings established by the Patent Cooperation Treaty.
Ce pouvoir s'applique également à toute procédure instituée par le Traité de coopération en matière de brevets.

[X] Weitere Vertreter sind auf einem gesonderten Blatt angegeben. / Additional representatives indicated on supplementary sheet.
Les autres mandataires sont mentionnés sur une feuille supplémentaire.

5 [X] Untervollmacht kann erteilt werden. / Sub-authorisation may be given. / Le pouvoir pourra être délégué.

6 [X] Bitte die gelbe Kopie, ergänzt um die Nr. der allgemeinen Vollmacht, an den Vollmachtgeber zurücksenden.
Please return the yellow copy, supplemented by the General Authorisation No., to the authorisor.
Prière de renvoyer la copie jaune au mandant, munie du n° du pouvoir général.

Ort/Place/Lieu Bagsværd Datum / Date 17.11.2000

Unterschrift(en) / Signature(s)

Arne W. Schmidt

Per Falholt

7 Das Formblatt muß vom (von den) Vollmachtgeber(n) (bei juristischen Personen vom Unterschriftsberechtigten) eigenhändig unterzeichnet sein. Nach der Unterschrift bitte den (die) Namen des (der) Unterzeichneten mit Schreibmaschine wiederholen (bei juristischen Personen die Stellung des Unterschriftsberechtigten innerhalb der Gesellschaft angeben).

The form must bear the personal signature(s) of the authorisor(s). (In the case of legal persons, that of the officer empowered to sign). After the signature, please type the name(s) of the signatory(ies) adding, in the case of legal persons, his (their) position within the company.

Le formulaire doit être signé de la propre main du (des) mandant(s) (dans le cas de personnes morales, de la personne ayant qualité pour signer). Veuillez ajouter à la machine après la signature, le (les) nom(s) du (des) signataire(s) en mentionnant, dans le cas de personnes morales, ses (leurs) fonctions au sein de la société.

M21-11-00

Novozymes A/S
Patents
Krogshøjvej 36
DK-2880 Bagsværd
DENMARK

Eine Mitteilung über die Registrierung der allgemeinen Vollmacht gelangt nicht von Amts wegen zu den Akten der Anmeldungen, für die der Bevoll-mächtigte als Vertreter bestellt ist oder bestellt wird. **Falls der Bevollmächtigte bereits für eine oder mehrere Anmeldungen als Vertreter bestellt ist und die vorliegende allgemeine Vollmacht hierfür verwenden will, wird er daher gebeten, zu der (den) betreffenden Anmeldung(en) möglichst umgehend die Inanspruchnahme und die Nr. der allgemeinen Vollmacht dem EPA mitzuteilen. Diese Mitteilung ist in der Stückzahl der betreffenden Anmeldungen einzureichen (Regel 36 (4)).**

Die allgemeine Vollmacht eines (von mehreren) Bevollmächtigten erlischt, sobald der Vollmachtgeber oder der betreffende Bevollmächtigte - nicht ein anderer Bevollmächtigter das Erlöschen dem EPA München, Direktion 5.1.1, mitgeteilt hat. Die Mitteilung muß klar und eindeutig sein. Insbesondere genügt nicht einfach die Einreichung einer neuen allgemeinen Vollmacht. auf der betreffende Bevollmächtigte fehlt (Regel 101 (5) und (6)).

A communication regarding the registration of the general authorisation is not inserted as a matter of course in the files relating to the applications for which the authorisee is or is to be appointed as representative. **If the authorisee is already appointed as representative for one or more applications and wishes to use the general authorisation therefore, he is accordingly requested to notify such wish together with the General Authorisation No. for the application(s) concerned as soon as possible to the EPO. One copy of such notification must be filed for each application concerned (Rule 36 (4)).**

The general authorisation of one or more authorisees terminates as soon as the authorisor or the authorisee concerned - not another authorisee - has communicated the termination to the EPO in Munich (Directorate 5.1.1). The communication must be clear and unambiguous. It is not sufficient to file a new general authorisation omitting the name of the authorisee concerned (Rule 101(5) and (6)).

L'enregistrement du pouvoir général ne fait pas d'office l'objet d'un avis dans les dossiers des demandes pour lesquelles le mandataire a été ou sera constitué en tant que tel. **Aussi, lorsque le mandataire est déjà constitué en tant que tel pour une ou plusieurs demandes et qu'il désire en l'oc-currence faire usage du présent pouvoir général, est-il prié de communiquer dans les plus brefs délais cette intention à l'OEB ainsi que le n° du pouvoir général pour la (les) demande(s) concernée(s). Cette communication doit être faite en autant d'exemplaires qu'il y a de demandes concernées (règle 36 (4)).**

Le pouvoir général d'un (de plusieurs) mandataire(s) prend fin, pour le mandataire concerné, dès que sa cessation a été notifiée par le mandant ou par le mandataire lui-même, à l'exclusion d'un autre mandataire, à l'OEB à Munich, Direction 5.1.1. Cette notification doit être claire et sans équivoque. En particulier, il ne suffit pas de déposer simplement un nouveau pouvoir général dans lequel il n'est plus fait mention du mandataire concerné (règle 101(5) et (6)).

BY FAX

01.11.00

Novo Nordisk

European Patent Office
D-80298 Munchen
Germany
Att.: Cash & Account

Novo Nordisk A/S
Enzyme Business
Patents

Novo Allé
DK-2880 Bagsværd
Denmark

Phone: +45 44448888
Fax: +45 44426080

A/S Reg. No. 16201

Bagsværd, 02 November 2000

Our ref.: Helix-EPO

Relating to our account no.28030007

The Board of Directors of Novo Nordisk A/S has proposed a demerger of Novo Nordisk into a health care company (Novo Nordisk A/S) and an enzyme company (Novozymes A/S). This demerger will be presented to the shareholders at an extraordinary general meeting on 13 november 2000.

The reorganisation will consist of Novo Nordisk A/S transferring its activities within enzyme business to a newly established Danish limited liability company, Novozymes A/S, listed on the Copenhagen Stock Exchange.

As a consequence of this demerger, the patents and patent applications in the name of Novo Nordisk A/S (Enzyme Business Patents) shall from 14 november 2000 belong to Novozymes A/S.

Our Address will change 14 november 2000 from

Novo Nordisk A/S
Enzyme Business Patents
Novo Alle
2880 Bagsværd

to

Novozymes A/S
Patents
Krogshøjvej 36
DK 2800 Bagsværd, Denmark

In the event that you have questions or comments to the above, please do not hesitate to contact us.

Sincerely yours
Enzyme Business Patents

Jette Vesterdal Hansen

EPO - DG 1

21. 08. 2000

(55)

Eintritt in die regionale Phase vor dem EPA als Bestimmungsamt oder ausgewähltem Amt

Entry into the regional phase before the EPO as designated or elected Office

Entrée dans la phase régionale devant l'OEB agissant en qualité d'office désigné ou élu

Europäische Anmeldenummer oder, falls nicht bekannt, PCT-Aktenzeichen oder PCT-Veröffentlichungsnummer	European application number, or, if not known, PCT application or publication number	Numéro de dépôt de la demande de brevet européen ou, à défaut, numéro de dépôt PCT ou de publication PCT
	99911648.6 - - PCT/DK9900202	
Zeichen des Anmelders oder Vertreters (max. 15 Positionen)	Applicant's or representative's reference (max. 15 spaces)	Référence du demandeur ou du mandataire (15 caractères ou espaces au maximum)
	5570.205-EP, SLK	

1. Anmelder

☒ Die Angaben über den (die) Anmelder sind in der internationalen Veröffentlichung enthalten oder vom Internationalen Büro nach der internationalen Veröffentlichung vermerkt werden.

☐ Änderungen, die das Internationale Büro noch nicht vermerkt hat, sind auf einem Zusatzblatt angegeben.

Zustellanschrift
(siehe Merkblatt II, 1)

1. Applicant

Indications concerning the applicant(s) are contained in the international publication or recorded by the International Bureau after the international publication

Changes which have not yet been recorded by the International Bureau are set out on an additional sheet.

Address for correspondence
(see Notes II, 1)

1. Demandeur

Les indications concernant le(s) demandeur(s) figurent dans la publication internationale ou ont été enregistrées par le Bureau international après la publication internationale

Les changements qui n'ont pas encore été enregistrés par le Bureau international sont indiqués sur une feuille additionnelle.

Adresse pour la correspondance
(voir notice II, 1)

2. Vertreter

Name (Nur einen Vertreter angeben, der in das europäische Patentregister eingetragen und an den zugestellt wird)

Geschäftsanschrift

Telefon

Telefax **Telex**

2. Representative

Name (Name **only one** representative who will be listed in the Register of European Patents and to whom notification will be made)

Sten Lottrup Knudsen

Address of place of business

Novo Nordisk A/S
Enzyme Business Patents
Novo Allé
2880 Bagsværd, DENMARK

Telephone

+ 45 44 44 88 88

Fax **Telex**

+ 45 42 60 80

2. Mandataire

Nom (N'indiquer **qu' un seul** mandataire, qui sera inscrit au Registre européen des brevets et auquel signification sera faite)

Adresse professionnelle

Téléphone

Téléfax **Télex**

☒ Weitere(r) Vertreter auf Zusatzblatt

Additional representative(s) on additional sheet

Autre(s) mandataire(s) sur une feuille additionnelle

3. Vollmacht

☐ Einzelvollmacht ist beigefügt.

☒ Allgemeine Vollmacht ist registriert unter Nummer.

24307

☐ Allgemeine Vollmacht ist eingereicht, aber noch nicht registriert.

☐ Die beim EPA als PCT-Anmeldeamt eingereichte Vollmacht schließt ausdrücklich die regionale Phase ein

3. Authorisation

Individual authorisation is attached.

General authorisation has been registered under No:

A general authorisation has been filed, but not yet registered.

The authorisation filed with the EPO as PCT receiving Office expressly includes the regional phase.

3. Pouvoir

Un pouvoir spécial est joint.

Un pouvoir général a été enregistré sous le n° :

Un pouvoir général a été déposé, mais n'est pas encore enregistré.

Le pouvoir général déposé à l'OEB agissant en qualité d'office récepteur au titre du PCT s'applique expressément à la phase régionale

☒ **4. Prüfungsantrag**
Hiermit wird die Prüfung der Anmeldung gemäß Art. 94 EPÜ beantragt
Die Prüfungsgebühr wird (wurde) entrichtet.

Prüfungsantrag in einer zugelassenen Nichtamtssprache (siehe Merkblatt III, 6.2).

4. Request for examination
Examination of the application under Art. 94 EPC is hereby requested.
The examination fee is being (has been, will be) paid.

IPEA: EPO
Request for examination in an admissible non-EPO language (see Notes III, 6.2):

Hermed begæres prøvning i henhold til Art. 94

4. Requête en examen
Il est demandé que soit examinée la demande de brevet conformément à l'art. 94 CBE. Il est (a été, sera) procédé au paiement de la taxe d'examen.

Requête en examen dans une langue non officielle autorisée (voir notice III, 6.2):

☐ **5. Abschriften**
Zusätzliche Abschrift(en) der im ergänzenden europäischen Recherchenbericht angeführten Schriftstücke wird (werden) beantragt.

Anzahl der **zusätzlichen** Sätze von Abschriften

5. Copies
Additional copy (copies) of the documents cited in the supplementary European search report is (are) requested

Number of **additional** sets of copies

5. Copies
Prière de fournir une ou plusieurs copie supplémentaire des documents cités dans le rapport complémentaire de recherche européenne

Nombre de jeux **supplémentaires** de copies

6. Für das Verfahren vor dem EPA bestimmte Unterlagen

6.1 Dem Verfahren vor dem EPA als **Bestimmungsamt** (PCT I) sind folgende Unterlagen zugrunde zu legen.

☒ die vom Internationalen Büro **veröffentlichten Anmeldungsunterlagen** (mit **allen** Ansprüchen, Beschreibung und Zeichnungen), gegebenenfalls mit den geänderten Ansprüchen nach Art 19 PCT

☐ soweit sie nicht ersetzt werden durch die **in drei Stücken** beigefügten **Änderungen.**

Falls nötig, sind Klarstellungen auf einem Zusatzblatt einzureichen!

6.2 Dem Verfahren vor dem EPA als **ausgewähltem Amt** (PCT II) sind folgende Unterlagen zugrunde zu legen:

☒ die dem **internationalen vorläufigen Prüfungsbericht zugrunde gelegten Unterlagen,** einschließlich seiner eventuellen **Anlagen**
(Solche Anlagen müssen immer in drei Stücken beigefügt werden)

☐ soweit sie nicht ersetzt werden durch die **in drei Stücken** beigefügten **Änderungen.**

Falls nötig, sind Klarstellungen auf einem Zusatzblatt einzureichen!

☒ Sind dem EPA als mit der internationalen vorläufigen Prüfung beauftragten Behörde **Versuchsberichte** zugegangen, dürfen diese dem Verfahren vor dem EPA zugrunde gelegt werden.

6. Documents intended for proceedings before the EPO

6.1 Proceedings before the EPO as **designated Office** (PCT I) are to be based on the following documents.

the **application documents published** by the International Bureau (with **all** claims, description and drawings), where applicable with amended claims under Art. 19 PCT

unless replaced by the **amendments** enclosed **in triplicate.**

Where necessary, clarifications must be submitted on a separate sheet!

6.2 Proceedings before the EPO as **elected Office** (PCT II) are to be based on the following documents

the **documents on which the international preliminary examination report is based,** including its possible **annexes**
(Such annexes must always be filed in triplicate)

unless replaced by the **amendments** enclosed **in triplicate.**

Where necessary, clarifications must be submitted on a separate sheet!

If the EPO as International Preliminary Examining Authority has received **test reports,** these may be used as the basis of proceedings before the EPO

6. Pièces destinées à la procédure devant l'OEB

6.1 La procédure devant l'OEB agissant en qualité **d'office désigné** (PCT I) doit se fonder sur les pièces suivantes :

les **pièces de la demande publiée** par le Bureau international (avec **toutes** les revendications, la description et les dessins), éventuellement avec les revendications modifiées conformément à l'article 19 du PCT

dans la mesure où elles ne sont pas remplacées par les **modifications** jointes **en trois exemplaires.**

Le cas échéant, des explications doivent être jointes sur une feuille additionnelle!

6.2 La procédure devant l'OEB **agissant en qualité d'office élu** (PCT II) doit se fonder sur les pièces suivantes :

les **pièces sur lesquelles se fonde le rapport d'examen préliminaire international,** y compris ses **annexes** éventuelles
(De telles annexes sont toujours à joindre en trois exemplaires)

dans la mesure où elles ne sont pas remplacées par les **modifications** jointes **en trois exemplaires.**

Le cas échéant, des explications doivent être jointes sur une feuille additionnelle!

Si l'OEB, agissant en qualité d'administration chargée de l'examen préliminaire international, a reçu des **rapports d'essais,** ceux-ci peuvent constituer la base de la procédure devant l'OEB.

7. Übersetzungen
Beigefügt sind die nachfolgend angekreuzten Übersetzungen in einer der Amtssprachen des EPA (Deutsch, Englisch, Französisch).

7. Translations
Translations in one of the official languages of the EPO (English, French, German) are enclosed as crossed below:

7. Traductions
Vous trouverez, ci-joint, les traductions cochées ci-après dans l'une des langues officielles de l'OEB (allemand, anglais, français)

- *Im Verfahren vor dem EPA als **Bestimmungsamt oder ausgewähltem Amt** (PCT I + II):*

☐ Übersetzung der **ursprünglich eingereichten internationalen Anmeldung** (Beschreibung, Ansprüche, etwaige Textbestandteile in den Zeichnungen), der veröffentlichten Zusammenfassung, und etwaiger Angaben über Mikroorganismen nach Regel 13bis.3 und 13bis.4 PCT, **in drei Stücken**

☐ Übersetzung der **prioritätsbegründenden Anmeldung(en), in einem Stück**

- *In proceedings before the EPO as **designated or elected Office** (PCT I + II)*

Translation of the **international application** (description, claims, any text in the drawings) **as originally filed**, of the abstract as published and of any indication under Rule 13bis.3 and 13bis.4 PCT regarding micro-organisms, **in triplicate**

Translation of **the priority application(s), in one copy**

- *Dans la procédure devant l'OEB agissant en qualité d'**office désigné ou élu** (PCT I + II):*

Traduction de la **demande internationale telle que déposée initialement** (description, revendications, textes figurant éventuellement dans les dessins), de l'abrégé publié, et de toutes indications visées aux règles 13bis.3 et 13bis.4 du PCT concernant les micro-organismes, **en trois exemplaires**

Traduction de la (des) **demande(s) ouvrant le droit de priorité, en un exemplaire**

- ***Zusätzlich** im Verfahren vor dem EPA als **Bestimmungsamt** (PCT I)*

☐ Übersetzung der nach Art. 19 PCT **geänderten Ansprüche** nebst Erklärung, falls diese dem Verfahren vor dem EPA zugrunde gelegt werden sollen (siehe Feld 6), **in drei Stücken**

- ***In addition**, in proceedings before the EPO as **designated Office** (PCT I):*

Translation of **amended claims** and any statement under Art. 19 PCT, if the claims as amended are to form the basis for the proceedings before the EPO (see Section 6), **in triplicate**

- ***De plus**, dans la procédure devant l'OEB agissant en qualité d'**office désigné** (PCT I) :*

Traduction des **revendications modifiées** et de la déclaration faite conformément à l'article 19 du PCT, si la procédure devant l'OEB doit être fondée sur les revendications modifiées (voir le rubrique 6), **en trois exemplaires**

- ***Zusätzlich** im Verfahren vor dem EPA als **ausgewähltem Amt** (PCT II):*

☐ Übersetzung der **Anlagen zum internationalen vorläufigen Prüfungsbericht, in drei Stücken**

- ***In addition**, in proceedings before the EPO as **elected Office** (PCT II):*

Translation of any **annexes to the international preliminary examination report, in triplicate**

- ***De plus**, dans la procédure devant l'OEB agissant en qualité d'**office élu** (PCT II) :*

Traduction des **annexes du rapport d'examen préliminaire international, en trois exemplaires**

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8. Biologisches Material
☐ Die Erfindung bezieht sich auf bzw verwendet biologisches Material, das nach Regel 28 EPÜ hinterlegt worden ist

8. Biological material
The invention relates to and/or uses biological material deposited under Rule 28 EPC

8. Matière biologique
L'invention concerne et/ou utilise la matière biologique, déposée conformément à la règle 28 CBE

☐ Die **Angaben nach Regel 28(1)c) EPÜ** (falls noch nicht bekannt, die Hinterlegungsstelle und das (die) Bezugszeichen [Nummer, Symbole usw.] des Hinterlegers) sind in der internationalen Veröffentlichung oder in der gemäß Feld 7 eingereichten Übersetzung enthalten auf:

Seite(n) / Zeile(n)

The **particulars referred to in Rule 28(1)(c) EPC** (if not yet known, the depository institution and the identification reference(s) [number, symbols etc.] of the depositor) are given in the international publication or in the translation submitted under Section 7 on:

page(s) / line(s)

Les **indications visées à la règle 28(1)c) CBE** (si pas encore connues, l'autorité de dépôt et la (les) référence(s) d'identification [numéro ou symboles etc.] du déposant) figurent dans la publication internationale ou dans une traduction produite conformément à la rubrique 7 à la / aux

page(s) / ligne(s)

Die **Empfangsbescheinigung(en)** der Hinterlegungsstelle

The **receipt(s) of deposit** issued by the depositary institution

Le(s) **récépissé(s) de dépôt** délivré(s) par l'autorité de dépôt

☐ ist (sind) beigefügt

is (are) enclosed

est (sont) joint(s)

☐ wird (werden) nachgereicht

will be filed at a later date

sera (seront) produit(s) ultérieurement

☐ Verzicht auf die Verpflichtung des Antragstellers nach Regel 28(3) auf gesondertem Schriftstück

Waiver of the right to an undertaking from the requester pursuant to Rule 28(3) attached.

Renonciation, sur document distinct, à l'engagement du requérant au titre de la règle 28(3)

9. Nucleotid- und Aminosäuresequenzen

☒ Die nach Regeln 5.2 und 13ter PCT sowie Regel 104b (3a) EPÜ erforderlichen Unterlagen liegen dem EPA bereits vor

☐ Das schriftliche Sequenzprotokoll wird anliegend in einer Amtssprache des EPA nachgereicht.

☐ Das Sequenzprotokoll geht nicht über den Inhalt der Anmeldung in der ursprünglich eingereichten Fassung hinaus.

☐ Der vorgeschriebene maschinenlesbare Datenträger ist beigefügt.

☐ Die auf dem Datenträger gespeicherte Information stimmt mit dem schriftlichen Sequenzprotokoll überein

9. Nucleotide and amino acid sequences

The items necessary in accordance with Rules 5.2 and 13ter PCT and Rule 104b (3a) EPC have already been furnished to the EPO

The written sequence listing is furnished herewith in an official language of the EPO.

The sequence listing does not include matter which goes beyond the content of the application as filed

The prescribed machine-readable data carrier is enclosed.

The information recorded on the data carrier is identical to the written sequence listing.

9. Séquences de nucléotides et d'acides aminés

Les pièces requises selon les règles 5.2 et 13ter PCT et la règle 104ter (3bis) CBE ont déjà été déposées auprès de l'OEB

La liste de séquences écrite est produite ci-joint dans une des langues officielles de l'OEB

La liste de séquences ne contient pas d'éléments s'étendant au-delà du contenu de la demande telle qu'elle a été déposée.

Le support de données prescrit, déchiffrable par machine, est annexé.

L'information figurant sur le support de données est identique à celle que contient la liste de séquences écrite.

10. Benennungsgebühren

10.1 Benennungsgebühren werden für nachstehende **in der internationalen Anmeldung bestimmte Vertragsstaaten** des EPÜ entrichtet.

10. Designation fees

10.1 Designation fees are paid in respect of the following EPC **Contracting States designated in the international application** for a European patent:

10. Taxes de désignation

10.1 Les taxes de désignation sont acquittées pour ceux des **Etats contractants** de la CBE **désignés dans la demande internationale** qui sont indiqués ci-après:

☐	AT	Österreich	Austria	Autriche
☐	BE	Belgien	Belgium	Belgique
☐	CH/LI	Schweiz und Liechtenstein	Switzerland and Liechtenstein	Suisse et Liechtenstein
☐	CY	Zypern [1]	Cyprus [1]	Chypre [1]
☒	DE	Deutschland	Germany	Allemagne
☐	DK	Dänemark	Denmark	Danemark
☐	ES	Spanien	Spain	Espagne
☐	FI	Finnland	Finland	Finlande
☐	FR	Frankreich	France	France
☒	GB	Vereinigtes Königreich	United Kingdom	Royaume-Uni
☐	GR	Griechenland	Greece	Grèce
☐	IE	Irland	Ireland	Irlande
☐	IT	Italien	Italy	Italie
☐	LU	Luxemburg	Luxembourg	Luxembourg
☐	MC	Monaco	Monaco	Monaco
☒	NL	Niederlande	Netherlands	Pays-Bas
☐	PT	Portugal	Portugal	Portugal
☐	SE	Schweden	Sweden	Suède
☐	____	_____ [2]	_____ [2]	_____ [2]
☐	____	_____ [2]	_____ [2]	_____ [2]

☒ 10.2 Derzeit ist nicht beabsichtigt, Benennungsgebühren für die in Feld 10.1 nicht angekreuzten, aber in der internationalen Anmeldung bestimmten Vertragsstaaten des EPÜ zu entrichten. Insoweit wird auf die Zustellung einer Mitteilung nach Regel 85a(1) EPÜ verzichtet. Sofern diese Benennungsgebühren nicht bis zum Ablauf der in Regel 85a(2) EPÜ vorgesehenen Nachfrist entrichtet werden, wird beantragt, von einer Mitteilung nach Regel 69(1) EPÜ abzusehen

10.2 At present it is not intended to pay designation fees for the EPC Contracting States not marked with a cross under 10.1 but designated in the international application. No communication under Rule 85a(1) EPC in respect of these designation fees need be notified. If they have not been paid by the time the period of grace allowed in Rule 85a(2) EPC expires, it is requested that no communication be sent under Rule 69(1) EPC.

10.2 Il n'est pas actuellement envisagé d'acquitter les taxes de désignation pour les Etats contractants de la CBE qui ne sont pas cochés sous la rubrique 10.1, mais qui sont désignés dans la demande internationale. Le demandeur renonce ainsi à la notification prévue à la règle 85bis(1) CBE. Si ces taxes de désignation ne sont pas acquittées à l'expiration du délai supplémentaire prévu à la règle 85bis(2) CBE, il est demandé de s'abstenir d'envoyer une notification, établie conformément à la règle 69(1) CBE.

1) Nur möglich, falls in der internationalen Anmeldung am oder nach dem 1. April 1998 bestimmt

2) Vorgesehen für die Eintragung weiterer Vertragsstaaten des EPÜ, für die der PCT oder das EPÜ nach Drucklegung dieses Formblatts in Kraft tritt, und die in der internationalen Anmeldung für ein europäisches Patent bestimmt waren

1) Only possible if designated in the international application on or after 1 April 1998

2) Space for any other EPC Contracting States which may become PCT or EPC Contracting States after this form has been printed and which were designated for a European patent in the international application

1) Seulement possible, si désignée dans la demande internationale au 1er avril 1998 ou après cette date

2) Prévu pour l'inscription d'autres Etats contractants de la CBE à l'égard desquels le PCT ou la CBE entrera en vigueur après l'impression du présent formulaire et qui ont été désignés dans la demande internationale pour un brevet européen

☒ **11. Erstreckung des europäischen Patents**

Diese Anmeldung gilt auch als Erstreckungsantrag hinsichtlich aller **in der internationalen Anmeldung bestimmten Nicht-Vertragsstaaten** des EPÜ, mit denen bei Einreichung der internationalen Anmeldung »Erstreckungsabkommen« in Kraft waren*
Die Erstreckung wird jedoch nur wirksam, wenn die vorgeschriebene Erstreckungsgebühr entrichtet wird.
Der Anmelder beabsichtigt, die Erstreckungsgebühr für die nachfolgend angekreuzten Staaten zu entrichten:

11. Extension of the European patent

This application is also considered as being a request for extension to all the **non-Contracting States** to the EPC **designated in the international application** with which "extension agreements" were in force on the date of filing the international application*
However, the extension only takes effect if the prescribed extension fee is paid.
The applicant intends to pay the extension fee for the States marked with a cross below.

11. Extension des effets du brevet européen

La présente demande est également réputée demande d'extension à tous les **Etats non contractants de** la CBE **désignés dans la demande internationale**, avec lesquels existaient, lors du dépôt de la demande, des «accords d'extension»*. **Toutefois, l'extension ne produit ses effets que si la taxe d'extension prescrite est acquittée.**
Le demandeur se propose actuellement d'acquitter la taxe d'extension pour les Etats dont le nom est coché ci-après:

☐	SI	Slowenien (* ab 1. März 1994)	Slovenia (* as of 1 March 1994)	Slovénie (* à compter du 1er mars 1994)
☐	LT	Litauen (* ab 5. Juli 1994)	Lithuania (* as of 5 July 1994)	Lituanie (* à compter du 5 juillet 1994)
☐	LV	Lettland (* ab 1 Mai 1995)	Latvia (* as of 1 May 1995)	Lettonie (* à compter du 1er mai 1995)
☐	AL	Albanien (* ab 1. Februar 1996)	Albania (* as of 1 February 1996)	Albanie (* à compter du 1er février 1996)
☐	RO	Rumänien (* ab 15. Oktober 1996)	Romania (* as of 15 October 1996)	Roumanie (* à compter du 15 octobre 1996)
☐	MK	Ehemalige jugoslawische Republik Mazedonien (* ab 1 November 1997)	Former Yugoslav Republic of Macedonia (* as of 1 November 1997)	Ex-République yougoslave de Macédoine (* à compter du 1er novembre 1997)
☐	____	_____ 1)	_____ 1)	_____ 1)

1) Platz für Staaten, mit denen »Erstreckungsabkommen« nach Drucklegung dieses Formblatts in Kraft treten und die in der internationalen Anmeldung bestimmt waren

1) Space for States with which "extension agreements" enter into force after this form has been printed and which were designated in the international application

1) Prévu pour des Etats à l'égard desquels des «accords d'extension» entreront en vigueur après l'impression du présent formulaire et qui ont été désignés dans la demande internationale

12. Automatischer Abbuchungsauftrag ***(Nur möglich für Inhaber von beim EPA geführten laufenden Konten)***

☒ Das EPA wird beauftragt, nach Maßgabe der Vorschriften über das automatische Abbuchungsverfahren fällige Gebühren und Auslagen vom untenstehenden laufenden Konto abzubuchen

Nummer des laufenden Kontos / Name des Kontoinhabers

_____

12. Automatic debit order ***(for EPO deposit account holders only)***

The EPO is hereby authorised, under the Arrangements for the automatic debiting procedure, to debit from the deposit account below any fees and costs falling due.

Deposit account number / Account holder's name

2803.0007 (Novo Nordisk A/S)

12. Ordre de prélèvement automatique ***(uniquement possible pour les titulaires de comptes courants ouverts auprès de l'OEB)***

Par la présente, il est demandé à l'OEB de prélever du compte courant ci-dessous les taxes et frais venant à échéance, conformément à la réglementation relative au prélèvement automatique

N° du compte courant / Nom du titulaire du compte

_____

☒ **13** Eventuelle **Rückzahlungen** auf das beim EPA geführte laufende Konto Nummer

_____

Name des Kontoinhabers

_____

13 Reimbursement, if any, to EPO deposit account number

2803.0007 (Novo Nordisk A/S)

Account holder's name

Novo Nordisk A/S

13. Remboursements éventuels à effectuer sur le compte courant ouvert auprès de l'OEB numéro

_____

Nom du titulaire du compte

_____

14. Unterschrift(en) des (der) Anmelder(s) oder Vertreters

Ort / Datum

14. Signature(s) of applicant(s) or representative

Place / Date

Bagsværd, 17 August 2000

Sten L. Knudsen

Sten Lottrup Knudsen
Representative of Applicant

14. Signature(s) du (des) demandeur(s) ou du mandataire

Lieu / Date

Für Angestellte (Art. 133(3) EPÜ) mit allgemeiner Vollmacht:

Nr. _____

Name(n) des (der) Unterzeichneten bitte mit Schreibmaschine wiederholen. Bei juristischen Personen bitte auch die Stellung des (der) Unterzeichneten innerhalb der Gesellschaft eintragen

For employees (Art. 133(3) EPC) having a general authorisation:

No. 24307

Please type name(s) under signature(s). In the case of legal persons, the position of the signatory within the company should also be typed

Pour les employés (art. 133(3) CBE) disposant d'un pouvoir général :

N° _____

Veuillez faire figurer le nom dactylographié sous la signature. Si ce nom désigne une personne morale, ajouter la mention dactylographiée de la position occupée par le signataire au sein de la société

European Patent Application No. 99911648.6 - PCT/DK99/00202

ADDITIONAL SHEET

Novo Nordisk

Novo Nordisk A/S
Enzyme Business
Patents

Novo Allé
DK-2880 Bagsvaerd
Denmark

Phone: +45 44448888
Fax +45 44426080

A/S Reg. No. 16201

Additional representatives

See General Authorisation No. 24307

EPO - DG 1

21. 08. 2000

(55)

Bagsværd, 17 August 2000

Sten L. Knudsen

Sten Lottrup Knudsen, representative of applicant

20. 07. 2000

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

15

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
5570-WO,SLK		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/DK99/00202	07/04/1999	08/04/1998

International Patent Classification (IPC) or national classification and IPC
C11B3/00

Applicant
NOVO NORDISK A/S

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 4 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 2 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
20/08/1999	21.12.00

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INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/DK99/00202

I. Basis of the report

1. This report has been drawn on the basis of (*substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.*):

Description, pages:

1-17	as originally filed

Claims, No.:

1-9	as received on	28/02/2000	with letter of	24/02/2000

Drawings, sheets:

1,2	as originally filed

2. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:
- ☐ the drawings, sheets:

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-9
	No:	Claims	
Inventive step (IS)	Yes:	Claims	1-9
	No:	Claims	
Industrial applicability (IA)	Yes:	Claims	1-9
	No:	Claims	

2. Citations and explanations

see separate sheet

Re Item V

Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. The present claims 1 to 9 are novel and inventive as required by Article 33(2,3) PCT.

Document D1 US-A-5 264 367 discloses in claims 1 t o14 and in column 3, lines 33 to 37 and in example 1 a process for reducing the content of phosphorus components in an edible oil.
The amounts of water used in the present application to obtain higher reduction in phosphorous components is not disclosed.

The present claims 1 to 9 are also novel and inventive in view of document D2 abstract of JP-A-215 3 997. The subject-treatment is performed with a phospholipase and a small amount of water.

However, in the present application the used amount of water is smaller and the obtained result is different.

CLAIMS 20. 07. 2000

1. A process for reducing the content of phosphorus containing components in an edible oil having from 50 to 10,000 part per million (ppm) of phosphorus content, which method comprises

a) emulsifying an aqueous solution of a phospholipase A1 (PLA1), phospholipase A2 (PLA2), or phospholipase B (PLB) in the oil using from 0.01 to 0.5 percent of water by weight of the oil,

b) contacting the oil with the emulsified phospholipase at a pH from 1.5 to 8 for 0.5-12 hours until the phosphorus content of the oil is reduced to less than 12 ppm, and then

c) separating the aqueous phase from the treated oil.

2. A process for reducing the content of phosphorus containing components in an edible oil having from 50 to 10,000 part per million (ppm) of phosphorus content, which method comprises

a) adjusting to a pH from 3 to 6 and emulsifying an aqueous solution of a phospholipase A1 (PLA1), phospholipase A2 (PLA2), or phospholipase B (PLB) in the oil using from 0.01 to 0.5 percent of water by weight of the oil,

b) contacting the oil with the emulsified phospholipase until the phosphorus content of the oil is reduced to less than 12 ppm, and then

c) separating the aqueous phase from the treated oil.

3. The process of claim 1 or 2, wherein the oil is an oil from which mucilage has previously been removed and which has a phosphorus content from 50 to 250 ppm.

4. The process of any of claims 1-3, wherein the phospholipase is a phospholipase obtained from a microorganism, preferably a filamentous fungus, a yeast, or a bacterium.

5. The process of claim 4, wherein the filamentous fungus is a species within the genus *Fusarium*, such as a strain of *F. culmorum*, F. *heterosporum*, *F. solani*, or in particular a strain of *F. oxysporum*.

6. The process of claim 4, wherein the filamentous fungus is a species within the genus *Aspergillus*, such as a strain of *Aspergillus awamori*, *Aspergillus foetidus*, *Aspergillus japonicus*, *Aspergillus niger* or in particular *Aspergillus oryzae*.

7. The process of any preceding claim, wherein the phospholipase is substantially independent of Ca^{2+} concentration measured as relative phospholipase activity at 5 mM EDTA and 5mM Ca^{2+} in a phospholipase activity assay measuring release of free fatty acids from lecithin in a buffer comprising 2% lecithin, 2% Triton X-100, 20 mM citrate, pH 5; incubated for 10 min. at 37°C followed by stop of reaction at 95°C for 5 min., wherein the ratio of relative phospholipase activity at 5mM EDTA/5 mM Ca^{2+} is greater than 0.25, more preferably greater than 0.5.

8. The process of any preceding claim, wherein the phospholipase is capable of releasing at least 7 µmol of free fatty acid/min./mg enzyme; more preferably at least 15 µmol of free fatty acid/min./mg enzyme; measured as phospholipase activity in an assay measuring release of free fatty acids from lecithin in a buffer comprising 2% lecithin, 2% Triton X-100, 20 mM citrate, pH 5; incubated for 10 min. at 37°C followed by stop of reaction at 95°C for 5 min..

9. The process of any of the preceding claims, wherein the phospholipase is a polypeptide selected from the group consisting of:

a) a polypeptide having an amino acid sequence as shown in positions 31-346 of SEQ ID NO 1;

b) a polypeptide having an amino acid sequence as shown in position 31-303 of SEQ ID NO 1;

c) a polypeptide which is at least 70 % homologous with the polypeptide defined in (a), or (b); and

d) a fragment of (a), (b) or (c).